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ORIGINAL PAPER

Bacterial and archaeal communities in the acid pit lake sediments of a chalcopyrite mine

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Abstract Bacterial and archaeal community structures and diversity of three different sedimentary environments (BH1A, BH2A and BH3A) in the acid pit lake of a chalcopyrite mine at Touro (Spain) were determined by 16S rRNA gene PCR-DGGE and sequencing of clone libraries. DGGE of bacterial and archaeal amplicons showed that the sediments harbor different communities. Bacterial 16S rRNA gene sequences were assigned to *Acidobacteria*, *Actinobacteria*, *Cyanobacteria*, *Planctomycetes*, *Proteobacteria*, *Chloroflexi* and uncultured bacteria, after clustering into 42 operational taxonomic units (OTUs). OTU 2 represented approximately 37, 42 and 37 % of all sequences from sediments BH1A, BH2A and BH3A, respectively, and was phylogenetically related to uncultured *Chloroflexi*. Remaining OTUs were phylogenetically related to heterotrophic bacteria, including representatives of *Ferrithrix* and *Acidobacterium* genera. Archaeal 16S rRNA gene sequences were clustered into 54 OTUs. Most of the sequences from the BH1A sediment were assigned to *Euryarchaeota*, whereas those from BH2A sediment were assigned to *Crenarchaeota*. The majority of the sequences from BH3A sediment were assigned to unclassified *Archaea*, and showed similarities to uncultured and unclassified environmental clones. No sequences related to *Acidithiobacillus* and *Leptospirillum*, commonly associated with acid mine drainage, were detected in this study.

Keywords 16S rRNA · Acid mine drainage · Chalcopyrite · Copper mine · Microbial diversity

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Introduction

Pit lakes are common in coal and metal sulfide open cut mining areas, and frequently originated from acid mine drainage (AMD), which is characteristically enriched in heavy metals such as Fe and Cu, and potentially toxic to the environment. AMD occurs when metal sulfide minerals (mostly pyrite, FeS_2) are oxidized upon exposure to air and water, generating a solution with high concentrations of H^+ and soluble metals (Nordstrom 1982). The rates of AMD generation may be affected by several factors, including the availability of oxidant and the microbial populations colonizing the mineral surfaces.

Overall, Eqs. (1)–(3) describe the oxidation of pyrite (Nordstrom 1982; Nordstrom and Alpers 1999):

$$FeS_2 + 7/2O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (1)$$

$$Fe^{2+} + 1/4O_2 + H^+ \rightarrow Fe^{3+} + 1/2H_2O \quad (2)$$

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+. \quad (3)$$

When O_2 is limiting and pH is low, ferric ion may oxidize pyrite generating more acidity (Eq. 4). In these environments, prokaryotic microorganisms, mostly

chemoautotrophic, play important roles in the oxidation of reduced forms of sulfur, contributing to AMD generation.

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (4)$$

AMD pit lakes are therefore extreme environments and may harbor unique microbial populations involved in the geochemistry of iron and sulfur. Several studies have been performed in the Iberian Pyritic Belt rivers, such as Tinto and Odiel, and in different AMDs in Spain and other parts of the world (Álvarez et al. 1993; Galán et al. 2003; Lee 2006; Nieto et al. 2007; Romero et al. 2007; Sánchez-Andrea et al. 2011; Sánchez-España et al. 2005) to understand metal geochemistry. However, studies on the microbial communities involved in the geochemistry of different metals in AMD systems, e.g., tailings, streams, biofilms, and pit lakes, are sparse (Auld et al. 2013; Hallberg et al. 2006; Rowe et al. 2007) and mostly concentrated in Chinese mines (Chen et al. 2013; Hao et al. 2010; Kuang et al. 2013; Yin et al. 2008).

The AMD pit lake of the Touro mine has been thoroughly chemically characterized. However, the microbial communities associated to the AMD pit lake sediments are unknown. Understanding the diversity of microorganisms that colonize AMD pit lakes and their functions may contribute to the development of new approaches for the bioremediation of AMD and AMD-contaminated sites. The goal of this study was to determine the bacterial and archaeal community structures and diversity in sediments from the AMD pit lake of the Touro mine and their possible roles in the geochemistry of iron and sulfur.

Materials and methods

Study area and sample collection

The study was carried out at the copper mine of Touro (Galicia, Spain, 42°52′24″N, 8°18′17″W) (Online Resource 1). The local geological substrate is formed mainly by schists and garnetiferous amphibolites, characterized by high concentrations of metal sulfides such as pyrite, pyrrhotite and chalcopyrite. The deposits of the metal sulfide are due to several volcanic intrusions into amphibolite. The spoil dump, mine slope and other surfaces of the quarry conserve a variable content of metal sulfide, even after Cu extraction (Álvarez et al. 2010; 2011; Otero et al. 2012). These deposits were exploited as an open cast mine, which occupies an area of 390 ha. The mine operated until the 1980s and at present the mine slopes and dumps are in process of restoration (Online Resource 1).

During the period of Cu extraction, the slurry materials were concentrated in a waste mud pile comprising a surface area of 80 ha and 80 m depth (Online Resource 1). The AMD (pH <3) was formed by the percolation of water through the pyrite-rich slurry materials and accumulated in a pit lake at the base of the mine. In the pit lake, several minerals precipitate forming sediments rich in Fe and trace metals (Online Resource 1).

Three types of sediments were sampled for microbial community analysis, according to their visual characteristics (Online Resource 1). The strong red precipitated sediment, named BH1A, and the red sediment with green filamentous organisms, named BH2A, were collected at the bottom of the lake, whereas a crusty superficial sediment, named BH3A, was sampled at the edge of the pit lake. The distance between each point was approximately 1 m and the depth of the water column oscillated between 0.5 and 1.0 m, except for the BH3A site, where the water column was approximately 0.10 m. The sediment samples were collected in sterile tubes in duplicates, freeze-dried and stored at −80 °C until processing.

Climate data was obtained from the meteorological station of Santiago de Compostela, located 10 km SW of the Touro mine (MeteoGalicia 2013). The average air temperature in the sampling day was 10.7 °C (day maximum 17.7 °C, minimum 6.0 °C). The average relative humidity was 62 % with no precipitation in the previous 15 days before sampling.

Sediment characterization

Sediment pH and redox potential (Eh) were measured in situ using a pH-redox meter (Hanna Instruments, model HI 9025). Final readings of redox potential were corrected by the addition of +244 mV of a calomel reference electrode. Sediment samples were centrifuged at 12,500*g* and 4 °C to extract porewater, and freeze-dried subsequently. In the porewater, the concentrations of dissolved Fe^{2+} and Fe^{3+} were determined using the 1,10-phenanthroline method (Stucki 1981). The following determinations were performed in the sediment solid fraction: pH in water (1:2.5, v:v), total organic carbon (TOC), total S (TS) and total nitrogen (TN) using a LECO CNS-2000 elemental analyzer. Total concentrations of Fe and Cu were determined after extraction of 0.5 g of ground sample with 15 ml of a mixture of HNO_3:HCl:HF (9:3:3, v/v/v) and heating the mixture in a Ethos Plus (Milestone) microwave oven for 20 min (Otero et al. 2013). Exchangeable Fe and Cu were extracted with 1 M $MgCl_2$ at pH 7 for 30 min (Tessier et al. 1979). Total and exchangeable metals were measured by graphite furnace atomic absorption spectrometry (Perkin-Elmer 4110ZL).

DNA extraction

Metagenomic DNA was extracted from 0.5 g of sediment sample. Two independent extractions per replicate were performed using the Fast DNA Spin kit (MP Biomedicals, France) and Fast Prep FP101 instrument (Savant, USA), according to the manufacturer's instructions. DNA integrity was evaluated by electrophoresis in 1 % agarose gel run in 1× TAE buffer (Tris, Acetic Acid, EDTA) and stained with Sybr Green (Life Technologies, USA). Gel image capture was performed using a Storm densitometer (GE Healthcare, Brazil). DNA concentration was determined by fluorometry using a Qubit fluorometer (Life Technologies, USA) and the Quant-iT dsDNA BR kit (Life Technologies, USA).

Bacterial and archaeal 16S rRNA gene amplicon analyses

Equal amounts of metagenomic DNA extracted from the sediment samples (two replicates) were pooled and used as template for amplification. A fragment of the 16S rRNA gene was amplified from metagenomic DNA by polymerase chain reaction (PCR) using the universal primers PRBA338fGC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG C-3′) and PRUN518R (5′-ATT ACC GCG GCT GCT GG-3′) for *Bacteria*. For *Archaea*, the amplification was performed by nested PCR using primers ARCH 21F (5′-TTC YGG TTG ATC CYG CCR GA-3′, Y=C or T, R=A or G) and ARCH 958R (5′-YCC GGC GTT GAN TCC AAT T-3′) for the first amplification, and ARCH 340FGC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC CTA CGG GGY GCA SCA G-3′, Y=C or T, S=C or G) and ARCH 519R (5′-TTA CCG CGG CKG CTG-3′, K=G or T) for the second amplification.

Amplification reactions were performed in triplicate in 1× Taq Polymerase Buffer (Fermentas Life Sciences, Canada) containing 100 ng of template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Life Technologies, USA), 1.5 U recombinant Taq DNA Polymerase (Fermentas Life Sciences, Canada), and 25 ρmol of each primer. PCR amplification conditions were 5 min at 95 °C; 30 cycles 1 min at 92 °C, 1 min at 55 °C and 1 min at 72 °C; and final extension for 10 min at 72 °C for bacterial DNA, and 5 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C; and final extension for 10 min at 72 °C for archaeal DNA. DNA integrity and concentration were determined as described above.

Equal amounts of amplicons from three amplification reactions per sample were pooled and 300 ng analyzed by DGGE using 8 % (w/v) acrylamide:bisacrylamide (37.5:1, m:m) gels containing a 15–55 % linear gradient of formamide and urea (100 % denaturing solution contained 40 % formamide and 7 M urea) according to Muyzer et al. (1993). Electrophoresis was performed at 200 V constant and 60 °C, using a DCode System (BioRad, USA), in 1× TAE buffer. Gels were stained with Vistra Green (GE Healthcare Life Sciences, Brazil) and analyzed by densitometry, using a Storm densitometer (GE Healthcare Life Sciences, Brazil) and the program Diversity Database (BioRad, USA).

DGGE banding patterns representing the bacterial and archaeal community structures in the sediments were analyzed as discrete data (presence or absence of bands with the same mobility in the gel, *Rf*) using Hierarchical Clustering Analysis based on simple matching similarity matrices calculated with the Ward's algorithm and Euclidian distances (Systat, SPSS Inc).

Bacterial and archaeal 16S rRNA gene sequencing

Metagenomic DNA from sediments BH1A, BH2A and BH3A was amplified by PCR using the primers BAC 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and BAC 1541R (5′-AAG GAG GTG ATC CAG CCG CA-3′) for *Bacteria*, and ARCH 21F/ARCH 958R and ARCH 21F/ARCH 519R for *Archaea*. PCR conditions were as described above, except that the amount of primers was 10 ρmol each.

Amplicons were analyzed by electrophoresis on 1 % agarose gels in 1× TAE buffer and stained with Sybr Green (Life Technologies, USA). Amplicons with the expected sizes were purified with the Invisorb CleanUp kit (Invitek, Germany), and ligated to pGEM-T Easy vector (Promega, USA) at 4 °C overnight, according to the manufacturer's instructions. The ligation product was transformed into *E. coli* DH5α competent cells by heat shock and transformed cells plated on LB-agar, containing ampicillin (100 μg mL^{-1}) and X-Gal (5-Brome-4-chloro-3-indolyl-β-D-galactoside). A total of 192 colonies containing recombinant plasmids were selected for each sediment library and cells grown in liquid LB medium containing 100 μg ampicillin mL^{-1} medium at 37 °C overnight. Plasmids were extracted by the alkaline lyses method (Sambrook et al. 2001) and the inserts sequenced using an ABI 3100 Automatic Sequencer (Life Technologies, USA), the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Brazil) and primers for T7 (5′-TAA TAC GAC TCA CTA TAG GGC-3′) and SP6 (5′-ATT TAG GTG ACA CTA TAG AAT ACT C-3′) regions, following the manufacturer's instructions.

Sequence analyses, operational taxonomic units (OTUs) definition and richness and diversity indices estimations

Nucleotide sequences (reads) were trimmed for the removal of low-quality bases (quality parameter >20, i.e., less than one error in 100 nucleotides) and vector sequences using Phred program (Ewing and Green 1998). Chimeric sequences were detected using the Chimera-Check algorithm (Cole et al. 2009) and excluded from the dataset. Valid sequences were aligned using ClustalX2 software (Thompson et al. 1997), setting gap opening penalty to 10 and gap extension penalty to 0.1 for pairwise and multiple alignments, and a Jukes–Cantor distance matrix was calculated by DNAdist (Felsenstein 1989). Sequences were clustered into OTUs using DOTUR (Schloss and Handelsman 2005), considering $d = 0.03$ for species definition. OTU richness was estimated using ACE and Chao-1 nonparametric estimators, and Shannon and reciprocal of Simpson's diversity indices were estimated using SPADE (Chao and Shen 2003).

Taxonomical affiliation of the sequences was performed using the Classifier tool of the Ribosomal Database Project II (http://rdp.cme.msu.edu/), with a confidence level of 80 %, naïve Bayesian algorithm and taxonomic hierarchy RDP 16S rRNA training set 9 (Wang et al. 2007). The sequences were also compared with the National Center for Biotechnology Information (NCBI) nucleotide database (NT/NR) using the MegaBlast (Altschul et al. 1990). Neighbor-joining phylogenetic trees with the most representative OTUs were constructed based on Kimura-2 algorithm and 1,000 bootstrap replicates using MEGA 5 (Tamura et al. 2011). The best sequence match of each OTU in Megablast (database NT/NR) was included in the phylogenetic tree as a reference (Altschul et al. 1990).

Multiple comparisons among the 16S rRNA gene clone libraries were performed using the S-Libshuff algorithm (Schloss et al. 2004).

The nucleotide sequences used in this study have been deposited in the Genbank database under the accession numbers KC746537 to KC747003.

Results

Sediments characterization

The three sediments sampled showed extremely acidic and oxic conditions, with pH ranging from 2.2 to 3.5, redox potential higher than 700 mV, and high concentrations of Fe^{2+} and Fe^{3+} in the porewater (Table 1). In the sediment BH1A, the concentrations of TOC, TS and TN were lower than in BH2A and BH3A. Total concentrations of Fe ranged from 39 to 47 %, and Cu from 100 to 120 mg kg^{-1} (Table 1). The total Cu concentration in the sediments was 2–3 times higher than in natural soils over amphibolite rocks (49 ± 31 mg kg^{-1}, according to Macías and Calvo de Anta 2009). The concentration of exchangeable Fe and Cu in the sediments was much lower than total concentration, but potentially more toxic since the exchangeable fraction presents high mobility and bioavailability in soils and sediments.

Table 1 Physical–chemical characteristics and concentration of total organic C (TOC), total N, total S, and total and exchangeable metals in the sediments sampled at the pit lake of the Touro mine

Parameter	BH1A	BH2A	BH3A
pH	2.1	2.3	3.4
Eh (mV)	710	704	706
TOC (%)	1.6	3.0	5.6
Total N (%)	0.13	0.23	0.22
Total S (%)	3.6	4.8	4.5
Total Fe (%)	47.2	39.6	41.6
Total Cu (mg kg^{-1})	270	102	196
Exchangeable Fe (mg kg^{-1})	547	972	161
Exchangeable Cu (mg kg^{-1})	1.57	1.67	1.19
Fe^{2+} porewater (mg l^{-1})	140	96.5	67.9
Fe^{3+} porewater (mg l^{-1})	340	245	195

Data are means of two replicates

Bacterial community structure and diversity

Based on the PCR-DGGE banding patterns, the sediments sampled showed distinct bacterial community structures and the bacterial community of sediments, BH2A and BH3A were more similar to each other as compared to sediment BH1A (Fig. 1a).

A total of 246 valid bacterial 16S rRNA gene sequences, 87 for BH1A, 76 for BH2A and 83 for BH3A, were obtained and used for further analyses. The majority of the sequences were assigned to the phylum *Chloroflexi*. Representatives of the phyla *Actinobacteria*, *Planctomycetes* and *Proteobacteria* were detected in all samples, whereas *Acidobacteria* was detected only in BH1A, and *Cyanobacteria* only in BH2A and BH3A. Nearly 6 % of BH1A and BH3A sequences were classified only at the *Bacteria* domain level (Table 2).

Bacterial richness (Chao-1 and ACE estimators) and diversity (Shannon and reciprocal of Simpson's) indices were not statistically different among the three sediments ($P < 0.05$) (Table 2), even though the structures of their communities were statistically different, based on S-Libshuff analyses ($P = 0.01$) (data not shown). Calculated sampling coverage was in the range of 88–90 %, although

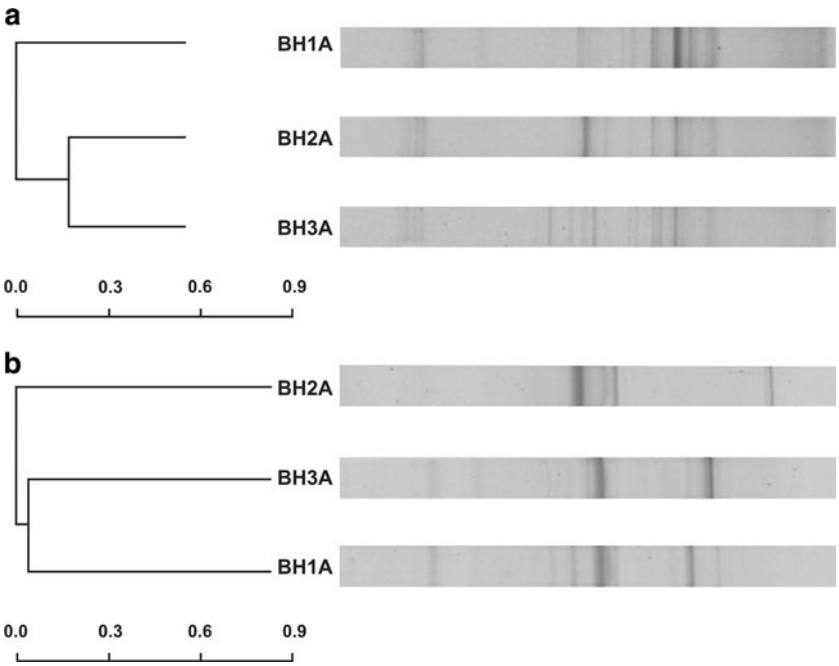


Fig. 1 Hierarchical clustering based on the profiles of bacterial (**a**) and archaeal (**b**) 16S rRNA gene amplicons from pit lake sediments BH1A, BH2A and BH3A

rarefaction curves suggested the need of an additional sequencing effort for covering most of the bacterial diversity (Online Resource 2a).

Valid sequences were clustered into 42 OTUs ($d = 0.03$). The complete list of the sequences comprising each OTU is shown in Online Resource 3. Only 6 OTUs were shared among all libraries and 9, 7 and 13 OTUs were exclusive of libraries BH1A, BH2A and BH3A, respectively. Phylogenetic analyses indicated the presence of a small dominant group of bacteria (Fig. 2) represented by OTU 2, which showed approximately 37, 42 and 37 % abundance in sediments BH1A, BH2A and BH3A, respectively. The clade containing OTUs 2, 3, 4, 5, 6, 10, 17 and 19 represented approximately 82, 56 and 64 % of the valid sequences for sediments BH1A, BH2A and BH3A, respectively (Fig. 2), and showed similarity to uncultured bacteria from the Nanshan waste ore deposits on the Mountain Xiang in China (access DQ453119, Hao et al. 2007), a volcanic deposit in Hawaii (AY917600, Gomez-Alvarez et al. 2007), unvegetated soil environments on Signy Island in Antartic (EF221273, Yergeau et al. 2007), and *Ktedonobacteria* Hsw-67 (*Chloroflexi*) from underground coal mine fire vent soil in China (GU237174, Xu and Zeng 2009, unpublished).

Less representative OTUs were mainly related to heterotrophic bacteria. OTUs 1 and 28, representing 14 % of BH2A sequences, showed similarity to *Mycobacterium* sp (AF236834, Leclerc et al. 2000) and *Mycobacterium shimoidei* (X82459, Boettger 2003, unpublished). OTU 22, representing 9 % of the BH2A sequences, was phylogenetically related to an uncultured *Planctomycetacia* (EF073417, Jangid et al. 2008). OTUs 29 and 36, representing 6 % of BH3A sequences, showed similarity to an uncultured *Gemmata* from sediments of an acid mine pit lake (FJ228382, Meier et al. 2009, unpublished). OTUs 27, 30 and 31, representing approximately 1 and 7 % of BH2A and BH3A sequences, respectively, showed similarity to an uncultured cyanobacterium clone (EF219700, Yergeau et al. 2007).

OTU 21 (5 % of the BH2A sequences) showed similarity to the obligate heterotrophic *Ferrithrix thermotolerans* (AY140237, Johnson et al. 2003), which is able to accelerate the oxidation of pyrite in the presence of yeast extract and dissimilatory reduction of ferric iron under anoxigenic conditions (Johnson et al. 2009) at moderate thermophilic conditions. This OTU also showed similarity to an uncultured bacterium from copper mine AMD (DQ458034). OTU 16 (2.3 % of the BH1A sequences) showed similarity to *Acidobacterium capsulatum* (CP001472, Ward et al. 2009), an acidophilic chemoorganotrophic isolated from acidic mineral environments (Kishimoto et al. 1991).

None of the detected sequences showed similarity to bacteria commonly associated with AMD, such as *Acidithiobacillus* and *Leptospirillum*.

Archaeal community structure and diversity

Similar to bacteria, the sediments showed distinct archaeal community structures, based on PCR-DGGE banding patterns (Fig. 1b). However, the levels of dissimilarity were higher, as compared to the ones observed for the bacterial

Table 2 Estimated richness, diversity indices for bacterial and archaeal communities and relative abundance of phylogenetic groups of pit lake sediments BH1A, BH2A and BH3A

	BH1A	BH2A	BH3A
Bacteria			
Richness/diversity index			
Chao-1[a]	26.1 (19.8; 53.6)	40.5 (19.6; 183.2)	28.1 (22.7; 50.4)
ACE[a]	30.6 (21.8; 60.2)	44.0 (20.7; 183.8)	33.1 (25.0; 58.1)
Simpson's reciprocal (1/*D*)[b]	3.9 (0.1; 9.0)	5.1 (3.4; 9.6)	2.9 (2.1; 4.9)
Shannon (*H*)[b]	1.9 (1.6; 2.2)	2.1 (1.9; 2.4)	1.9 (1.5; 2.2)
Coverage (%)[c]	89.7	90.8	88.0
Number of sequences[d]	87	76	83
Number of OTUs[e]	18	16	21
Phylogenetic group[f]			
Acidobacteria	2.3 %	ND	ND
Actinobacteria	8.0 %	30.3 %	13.3 %
Cyanobacteria/chloroplast	ND	1.3 %	7.2 %
Planctomycetes	1.1 %	10.5 %	8.4 %
Proteobacteria	1.1 %	1.3 %	2.4 %
Chloroflexi	81.6 %	56.6 %	62.7 %
Unclassified bacteria	5.7 %	ND	6.0 %
Archaea			
Richness/diversity index			
Chao-1[a]	39.3 (23.0; 122.2)	6.5 (6.0; 14.4)	133.5 (40.0; 686.3)
ACE[a]	43.8 (24.7; 125.6)	7.0 (6.1; 17.1)	141.0 (42.7; 683.3)
Simpson's reciprocal (1/*D*)[b]	8.6 (5.8; 16.6)	3.0 (1.9; 6.5)	5.8 (4.0; 10.8)
Shannon (*H*)[b]	2.4 (2.2; 2.6)	1.3 (1.1; 1.5)	2.3 (1.990; 2.596)
Coverage (%)[c]	89.4	98.6	76.2
Number of sequences[d]	85	73	63
Number of OTUs[e]	19	6	21
Phylogenetic group[f]			
Crenarchaeota	ND	91.8 %	1.6
Euryarchaeota	57.7 %	ND	ND
Unclassified Archaea	42.4 %	8.2 %	98.4 %

Values in parentheses represent the 95 % confidence intervals

ND not detected

[a] Nonparametric estimators of species richness: Chao-1 and ACE

[b] Diversity index: Simpson's reciprocal (1/*D*) and Shannon (*H*) maximum likelihood estimation

[c] Estimated sample coverage

[d] Number of valid sequences

[e] Estimated number of operational taxonomic units (>97 % similarity)

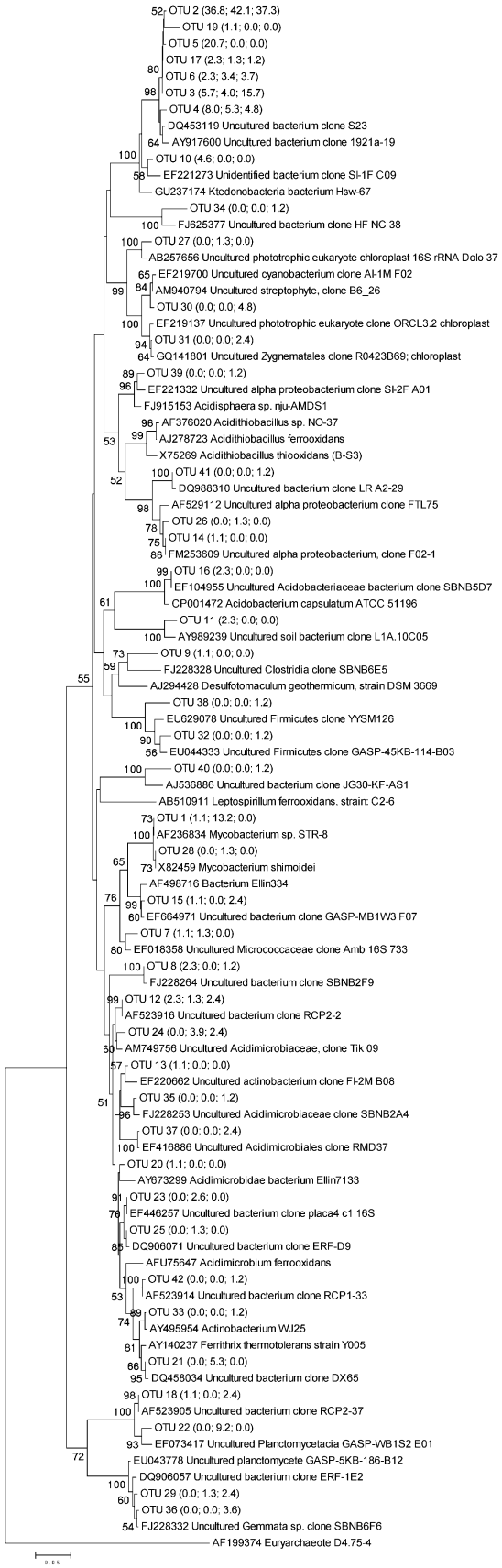
[f] Phylogenetic assignment using Classifier algorithm in RDP-II (Cole et al. 2009) at 80 % confidence threshold

communities. Similar results were observed by 16S rRNA gene cloning and sequencing. S-Libshuff analysis ($P = 0.01$) showed that the archaeal communities in the sampled sediments were statistically different (data not shown).

A total of 221 valid archaeal 16S rRNA gene sequences, 85 for BH1A, 73 for BH2A and 63 for BH3A, were obtained and used for further analyses. Approximately 58 % of the BH1A sequences were assigned to the phylum *Euryarchaeota* and 92 % of the BH2A sequences to *Crenarchaeota*. The remaining sequences, including approximately 98 % of BH3A sequences, showed no similarities to other sequences in the databases and were assigned to unclassified *Archaea* (Table 2).

Richness (Chao-1 and ACE estimators) and the Shannon diversity index were statistically lower in BH2A, as compared to BH1A and BH3A (Table 2). Estimated sample coverage ranged from approximately 76 % (BH3A) to 98 % (BH2A). Rarefaction curves indicated that most of the archaeal species were sampled (Online Resource 2b).

The valid sequences were clustered into 54 OTUs ($d = 0.03$). The complete list of sequences comprising each OTU is shown in Online Resource 3. All OTUs from BH2A sediment were clustered in a single clade and showed similarity to an uncultured *Crenarchaeotum* from a tidal flat sediment (AY396690, Kim et al. 2005), uncultured archaea from marine sediments (access DQ988474, GQ927558, GQ926246, Hu et al. 2006, unpublished) and a methane seep river sediment (FJ264795, Beal et al. 2009). Together, OTUs 29 and 30 represented approximately 77 % of all BH2A sequences (Fig. 3). The clade containing OTUs 3, 4, 6, 19, 20 and 21, represented approximately 33 % of BH1A sequences, and showed similarity to an uncultured archaeon from forest wetland impacted by rejected coal (AF523938, Brofft et al. 2002) and an uncultured *Thermoplasmatales* (*Euryarchaeota*) from



◀ **Fig. 2** Phylogenetic tree of bacterial 16S rRNA gene sequences from pit lake sediments BH1A, BH2A and BH3A. Tree was constructed using neighbor-joining. *Bootstrap values* higher than 50 % based on 1,000 replicates are indicated at the nodes. *Scale bar* represents 5 % nucleotide substitutions per position. Best match sequences from NCBI database were used as references. *Values* in the *parentheses* correspond to the relative abundance (%) of the OTU in BH1A, BH2A and BH3A sediment sample, respectively

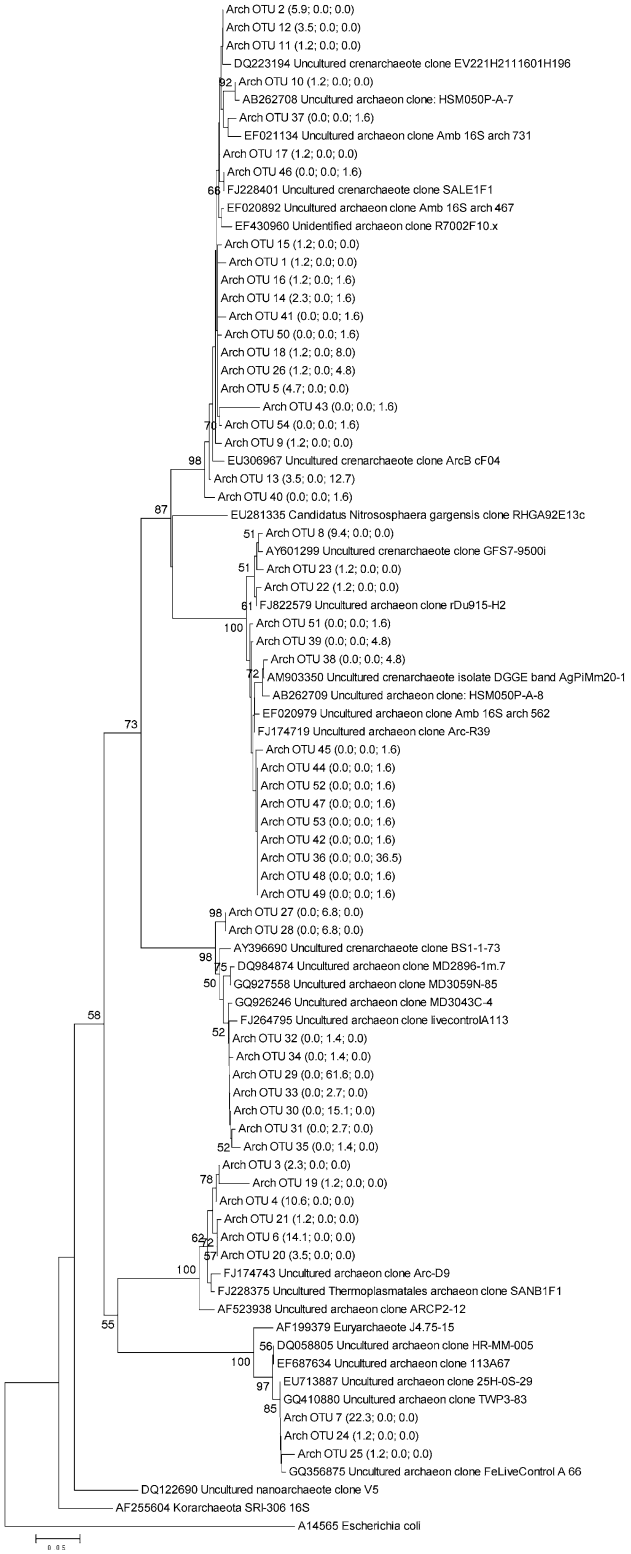
sediments of an acid mine pit lake (FJ228375, Meier et al. 2009, unpublished). OTUs 7, 24 and 25, representing approximately 25 % of BH1A sequences, showed similarity to uncultured archaea from a methane seep (GQ356875, Beal et al. 2005, unpublished) and volcano sediment underneath an iron-oxidizing mat (EF687634, Omoregie et al. 2008). OTU 36, representing approximately 36 % of BH3A sequences, showed similarity to uncultured archaea from an acidic red soil (FJ174719, Ying et al. 2010) and a rhizospheric soil (EF020979, Lesaulnier et al. 2008).

Discussion

The mining process at the Touro mine consisted basically in separating out chalcopyrite from pyrite, which becomes one of the major components of the slurry. The abiotic and biotic oxidation of pyrite (FeS_2) are the main geochemical processes that contribute to water acidification and increased solubility of Fe and Cu under oxic conditions (Álvarez et al. 1993). Pyrite can be oxidized by oxygen and ferric iron regenerated by microorganisms that catalyzes the oxidation of ferrous ions (Fowler et al. 1999; Silverman and Ehrlich 1964). The oxidation of Fe(II) to Fe(III) and reduced forms of sulfur by chemoautotrophic microorganisms results in proton generation and consequent acidification of mine drainage water, which has to be properly treated and disposed (Baker and Banfield 2003).

In our study, the exchangeable and total concentrations of Fe in the pit lake sediments indicated a strong influence of the percolation water from the slurry materials, which were enriched in pyrite and depleted in chalcopyrite due to the previous extraction of the former. Hence, high concentrations of dissolved Fe in the porewater were observed. In addition, due to oxic and extremely acidic conditions, the concentrations of Fe^{3+} were higher than Fe^{2+} in porewater. In contrast, the total concentration of Cu was lower, and the results of the sequential extraction showed that most of the Cu was associated with Fe-oxyhydroxides (data not shown) and, therefore, may not be readily bioavailable.

The physical–chemical analysis of the Touro AMD pit lake sediments also reveled higher concentrations of TOC,



◀ **Fig. 3** Phylogenetic tree of archaeal 16S rRNA gene sequences from pit lake sediments BH1A, BH2A and BH3A. Tree was constructed using neighbor-joining. *Bootstrap values* higher than 50 % based on 1,000 replicates are indicated at the nodes. *Scale bar* represents 5 % nucleotide substitutions per position. Best match sequences from NCBI database were used as references. *Values* in the *parentheses* correspond to the relative abundance (%) of the OTU in BH1A, BH2A and BH3A sediment sample, respectively

TS and TN in samples BH2A and BH3A than in BH1A. These results may be due to the presence of a layer of algae related to *Zygnematales*, based on the sequence of chloroplastic 16S rRNA gene (data not show), in BH2A or arrival of organic matter from the edges of the pit lake in BH3A (Online Resource 1).

In AMD, the richness of microbial species is normally low and limited by the number of possible energy-deriving reactions (Baker and Banfield 2003). In our study, the bacterial 16S rRNA gene sequences obtained were clustered into 42 OTUs assigned to *Acidobacteria*, *Actinobacteria*, *Cyanobacteria*, *Planctomycetes*, *Proteobacteria*, *Chloroflexi* and unclassified bacteria. The bacterial richness observed was comparable to the one observed in wetland impacted with rejected coal pile drainage water (Brofft et al. 2002), and abandoned lead–zinc mine tailings (Zhang et al. 2007), but greater than the richness observed in sulfide AMD (He et al. 2007) and in macroscopic filaments from the Tinto River (Spain) (García-Moyano et al. 2007).

The microbial community associated with the sediments of the Touro mine AMD pit lake, in our study, was highly dominated by few bacterial groups, since 19 % of the OTUs corresponded to approximately 82, 56 and 64 % of all 16S rRNA gene clone sequences from BH1A, BH2A and BH3A, respectively. The dominance of few bacterial species may be attributed to the acid and energetically limited mineral substrates in such an environment where autotrophic metabolism is predominant. The phylogenetic analysis of the dominant OTUs (Fig. 2) indicated similarities to *Chloroflexi*-related uncultured bacteria from AMD impacted environments and volcanic areas. The phylum *Chloroflexi* is ubiquitous and a limited number of cultured representatives is known (Yamada and Sekiguchi 2009). High dominance of *Choroflexi*-like organisms has also been observed in a Fe(III)-rich sediment from an AMD impacted area in the USA (Senko et al. 2008). However, the roles of *Chloroflexi* in such environments are still uncertain. Species such as *Chloroflexus* spp. and *Roseiflexus* spp. may inhabit microbial mats in neutral and alkaline geothermal springs and alternate between heterotrophic and photosynthetic metabolism (Klatt et al. 2013; Zarzycki et al. 2009). In addition, carbon fixation by acidophilic filamentous algae, such as *Zygnematales*, would contribute to the growth of heterotrophic iron reducers, as proposed by Rowe et al. (2007). Recently, Nancucheo and Barrie Johnson (2012) observed that heterotrophic acidophilic *Acidiphilium* and *Acidobacterium* spp. are able to metabolize monosaccharides produced by *Chlorella* and *Euglena* in AMD environments. Souza-

Egipsy et al. (2008) proposed that photosynthetic biofilms in the Tinto River might be involved either in iron oxidation, through the release of oxygen in the water, or reduction, providing organic carbon for iron-reducing heterotrophic acidophiles. The role of algae biofilms on iron metabolism in the Touro AMD pit lake sediments, however, is not known and further studies on the eukaryotic diversity and function would be necessary.

The occurrence of the obligate chemolithotrophic bacteria, such as *Acidithiobacillus ferrooxidans*, *A. thiooxidans* and *Leptospirillum ferrooxidans*, has been frequently reported in AMD (Schippers and Sand 1999; Schrenk et al. 1998). However, in our study, no sequences phylogenetically related to these genera have been detected, suggesting that they do not occur in these sediments or that their abundances are below the detection limits of the methodological approach used. It is possible that in AMDs with pH >3, moderately acidophilic iron/sulfur oxidizing bacteria play a major role in metal oxidation, minimizing the importance of extremely acidophilic oxidizers (Hallberg and Johnson 2003). The absence of *A. ferrooxidans* and *L. ferrooxidans* might also be attributed to the depletion of their substrates such as Fe(II) (Table 2). Corroborating this hypothesis, the sequential extraction of iron in our study showed that Fe-oxyhydroxides are more abundant than Fe-pyrite. Furthermore, in such environments, pyrite might be coated with the Fe-oxyhydroxides precipitates, limiting the access of bacteria to pyrite surface and the oxidation process (Huminicki and Rimstidt 2009).

In contrast to *Bacteria*, most *Archaea* detected in our study showed no similarity to known species, including genera frequently reported in AMD such as *Sulfolobus*, *Acidianus* and *Ferroplasma* (Baker and Banfield 2003). However, the archaeal 16S rRNA gene sequences detected in our study were similar to sequences detected in soil, deep sea, marine sediments, volcanic areas and AMD impacted sites, indicating the dominance of so far uncultured archaeal species commonly detected in environments under extreme conditions. From the available data, the functions of the archaeal species in the sediments of the Touro pit lake cannot be established, and further studies would be necessary to determine their functions in this environment.

Conclusion

The study of the prokaryotic diversity in the AMD pit lake of Touro is essential for the understanding of the geochemical transformations taking place and developing of new approaches for AMD remediation. Regardless of the spatial proximity, the sediments studied showed distinct bacterial and archaeal communities. A high dominance of *Chloroflexi*-like microorganisms was observed. Even though they may act as primary producers, contributing to the heterotrophic growth of acidophilic microorganisms, their functions still remain unclear. Most archaeal sequences showed similarity to uncultured and unidentified environmental archaea and their possible functions cannot be established.

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