

New insights into the microbiology of meromictic acidic pit lakes in the Iberian Pyrite Belt (Spain)

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Abstract

Over twenty opencast voids in abandoned mines of the Iberian Pyrite Belt (IPB, Southwest Iberian Peninsula) are now filled with low pH water containing elevated concentrations of soluble metals and other solutes such as sulfate. Few studies have examined the microbiology of acidic metal-mine pit lakes, even though they are a potential source of new species of acidophiles that could be harnessed for mineral bio-processing (biomining) or bioremediation of acidic mine waters. Pit lakes in the IPB are very diverse in size, depth, age and water composition, but they share a common geological framework. The majority are meromictic and display a permanent stratification pattern. Two of these meromictic pit lakes, Cueva de la Mora and Guadiana, were chemically and microbiologically characterized. Acidophilic heterotrophic bacteria and algae populated the mixolimnion in both lakes, the transition layers in both lakes were found to be the most microbially diverse, and where iron and sulfur transformations were more important. In contrast, no bacteria were isolated from the monimolimnion (the lowest anoxic layer). DNA was extracted from two water samples from the monimolimnion (at depths of 55 and 60 m) in Guadiana pit lake, 16S rRNA bacterial and archaeal genes amplified and microbial diversity assessed using terminal restriction enzyme fragment length polymorphism (T-RFLP). Clone libraries of 16S rRNA genes of *Archaea* and *Bacteria* from samples were constructed in order to identify unknown T-RFs. Results obtained in this work indicate that the monimolimnion of Guadiana is populated by different bacterial community to that of the upper layers of the lake. Archaeal communities appeared to be very similar in waters from different depths within Guadiana, and were also similar to that found in the Cueva de la Mora monimolimnion.

Key words: pit lake, acid mine drainage, acidophiles, archaea, bacteria

Introduction

The Iberian Pyrite Belt (IPB) is located in the south-west of the Iberian Peninsula, and extends from the south of Lisbon to the west of Seville. This famous mining district contains many massive sulfide deposits of Palaeozoic (Carboniferous) age classified as giant and supergiant (Sáez et al. 1999), being one of the largest massive sulfide provinces on Earth (Leistel et al. 1998). The IPB has been mined for centuries, since the metal ages up to the present day, with several mines still in operation (e.g. Aguas Teñidas). The most intense mining activity took place in the Modern age, especially between 1880 and 1980 leaving a legacy of mine wastes, old mine structures, and flooded opencast pits. In the Spanish part of the IPB alone, more than 30 pit lakes have formed as a result of the abandonment and subsequent flooding of the former opencast excavations (Sánchez-España et al. 2008, López-Pamo et al. 2009). Pit lakes in the IPB are very diverse in size, depth, age and water composition, but they share a common geological framework. The majority are meromictic and display a permanent stratification pattern, with the water column divided into an upper oxic layer (the mixolimnion), a bottom anoxic layer (the monimolimnion) and a transitional chemocline separating them (Sánchez-España et al. 2009) where redox transformations of iron and sulfur are most dynamic.

Two meromictic pit lakes, Cueva de la Mora and Guadiana, have been chemically and microbiologically characterized (Falagán et al. 2014, 2015). The mixolimnion is characterized by low pH (2.2 – 2.9) and

high redox potential ($\sim +800$ mV), whilst the monimolimnion is characterized by high pH (4 – 4.5) and low redox potential ($\sim +300$ mV). The chemocline is characterized by pronounced gradients of physico-chemical parameters where oxidized and reduced forms (as for example iron and sulfur) co-exist. These metal-mine pit lakes are rich in dissolved metals (e.g. Fe, Zn, Mn) and sulfate, which usually increase in concentration with depth.

Acidophilic heterotrophic bacteria and algae populated the mixolimnion in both lakes, though maximum numbers of phototrophs were found at depths in the water column where the incident solar radiation was only $\sim 2\%$ of that at the lake surface. This has been ascribed to large variations in phosphorus bioavailability in the water column. The transition layers in both lakes were found to be the most microbially diverse, and included iron- and sulfur-oxidizing autotrophs, iron-reducing heterotrophs and also, in the case of Cueva de la Mora, sulfate-reducing bacteria. Shallow sediments (recovered at 8 m depth) contained similar microbial populations in both lakes, though those located deep within Cueva de la Mora were notably different (Falagán et al. 2014, 2015).

The monimolimnetic microbial community was not studied in depth in previous work. Only archaea were detected in the lower layer of the Cueva de la Mora pit lake, while no DNA was amplified in the Guadiana pit lake monimolimnion. This paper reports new insights into the microbial composition of the anoxic layer of the Guadiana pit lake.

Methods

Site of study and sampling

The study was carried out in the Guadiana pit lake (Herrerías mine, Spain; 37°46'66"N, 6°49'31"W) located in the IPB. The Guadiana pit lake has a mixolimnion that can be subdivided into two layers: an epilimnion (0 – 8 m depth), which is aerobic and influenced by seasonal temperature changes, overlying an anoxic hypolimnion (8 – 20 m depth), within which all measured physico-chemical parameters showed no depth-related changes. The monimolimnion, which extends from 20 to 65 m, is characterized by relatively high pH 4.0 – 4.5 and low redox potential (+330 – +400 mV). The monimolimnion is divided in two sub-layers, all the physico-chemical parameters follow an increasing (e.g. temperature) or decreasing (e.g. redox potential) pattern up to a depth of 50 m, after which all of these parameters are constant down to the lake bottom. The vertical pattern is deeply influenced by the existence of mine galleries intersecting the former mine pit, as reported by Sánchez-España et al. (2014). The Guadiana pit lake monimolimnetic waters are rich in sulfate (~ 7.4 g/L, $\text{SO}_4^{2-}\text{-S}$) and dissolved metals (e.g. Mn, ~ 400 mg/L; Zn, ~ 300 mg/L), but the most dominant metal is iron (~ 7.6 g/L) which due to the anoxic conditions of the monimolimnion is mainly as ferrous iron (Falagán et al. 2014). This lake presents very high CO_2 concentration in the monimolimnion (caused by the dissolution of carbonate present in spilites and by mineralization) so that a controlled degasification process is undergoing (Boehrer et al. 2016).

The Guadiana pit lake was visited in October 2014, water samples were collected from 55 and 60 m depth with a horizontal sampling bottle. Water samples were filtered through sterile membrane filters (0.2 μm pore size) and stored at $< 20^\circ\text{C}$ until processed.

Biomolecular analyses

DNA was extracted from the filters using MoBio “ultraclean soil DNA isolation kits”, following the manufacturer’s instructions. Ribosomal RNA genes (bacterial and archaeal 16S rRNA genes) were amplified using the polymerase chain reaction (PCR): the primers used were for 16S rRNA bacterial DNA the 27F (Lane 1991) and the 1387R (Marchesi et al. 1998), for 16S rRNA archaeal DNA the 20F and the 915R (Kay et al. 2013).

T-RFLP analyses of amplified genes were carried out to assess the microbial diversity of samples. Amplified DNA was separately digested with three restriction enzymes (HaeIII, CfoI, and AluI), the lengths of the gene fragments were determined using capillary electrophoresis, and the T-RFs (terminal restriction fragments) identified by comparing them with those in the databank maintained at Bangor University.

Two clone libraries were constructed, one with the 55 m sample for *Bacteria* and the other with the 60 m sample for *Archaea*. PCR products were ligated using the pGEM® -T-Easy cloning vector system (Promega, Madison, WI), and the resulting plasmids were transformed into *Escherichia coli* strain

DH5 α , in accordance with the manufacturer’s instructions. Plasmid inserts that generated different distinct restriction enzyme fragment length polymorphism (RFLP) were purified (StrataPrep Plasmid Miniprep kit, Agilent technologies) and sequenced (Macrogen, Inc., South Korea), (Kay et al. 2013).

Resulting bacterial and archaeal clone sequences were aligned using BLASTN online software (National Centre of Biotechnology Information, NCBI) and compared with those contained in the GenBank database. Sequences were deposited in the GenBank database under different accession numbers (KX083694 – KX083698).

Results

Results indicated that the monimolimnion of Guadiana was populated by different bacterial community to that of the upper layers of the lake. Both water samples, taken at 55 m and 60 m depths, presented similar T-RFLP (fig. 1) profiles for bacterial 16S rRNA digests with the restriction enzyme CfoI. T-RFLP profiles were dominated by two main peaks at 63 ± 2 nt and at 578 ± 2 nt (CfoI digests).

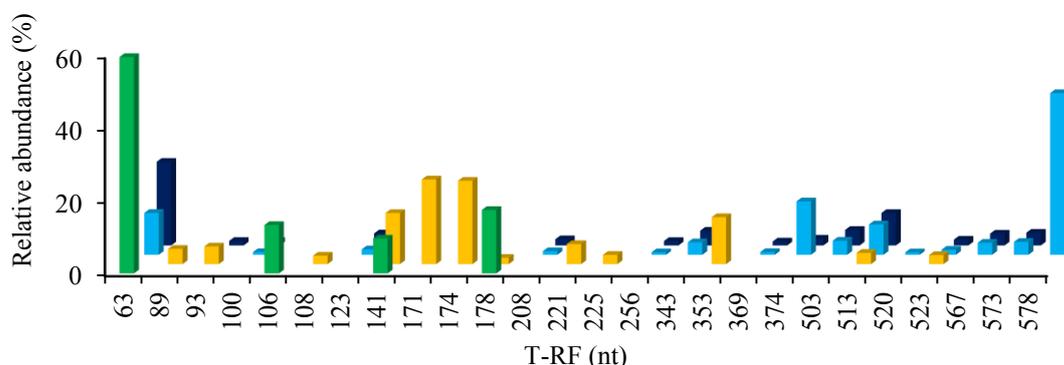


Figure 1 T-RFLP profiles of amplified bacterial 16S rRNA gene (digested with CfoI) of filtrates from the monimolimnion water samples taken at 55 m (■) and 60 m (■), of the Guadiana pit lake sampled in October 2014, and the hypolimnion (15 m deep; ■) and the epilimnion (7 m deep; ■) sampled on September 2012. The major peaks identified corresponded to *Metallibacterium scheffleri* (63 ± 2 nt), *Acidisphaera rubrifaciens* (177 ± 2 nt), MCF86 (144 ± 2 nt), and clones H55B-11, H55B-27, H55B-2-1 and H55B-2-7 (580 ± 2 nt).

Archaeal communities appeared to be very similar in waters from different depths within Guadiana, and were also similar to that found in the monimolimnion of the Cueva de la Mora pit lake (fig. 2). The archaeal 16S rRNA T-RFLP profile was dominated by two peaks at 62 ± 2 nt (60 m sample) and at 213 ± 2 nt (55 m sample). The 60 m sample appeared more diverse than the 55 m sample.

Analysis of the clone libraries has given insights into the indigenous microflora of the enigmatic anoxic layer of the Guadiana pit lake. Few distinct clones (four for bacteria and one for archaea) were identified by RFLP analysis of complete libraries. Two bacterial clones (H55B-2-1 and H55B-2-7) were 98% similar to *Thiomicrospira (T.) crunogena*^T, a sulfur-oxidizing bacterium that was first isolated from a hydrothermal vent in the Pacific Ocean, which was reported not to grow at pH < 5.0 (Jannasch et al. 1985). Clone H55B-11 was related 95% to *Gallionella ferruginea*^T. *G. ferruginea* is a neutrophilic iron-oxidizing chemolithotrophic betaproteobacteria found in micro-aerobic environments such as well waters (Hallbeck and Pedersen 1990), though related strains (which probably represent at least one distinct species) have also been found in acidic environments, such as acid mine drainage waters (Hallberg et al. 2006). Clone H55B-27 was very distantly related to any described species, being 92% and 91% related to *Hydrogenovibrio (H.) marinus*^T and *T. crunogena*^T, respectively.

The CfoI restriction enzyme cut the amplified 16S rRNA gene in positions 578 ± 2 nt for H55B-27, H55B-2-1 and H55B-2-7 clones and 582 ± 2 nt for the clone H55B-11, which corresponds the main peak (580 ± 2 nt) of the T-RFLP profile of bacterial 16S rRNA for samples of water from 55 and 60 m depths (fig. 1). Other peaks were not identified.

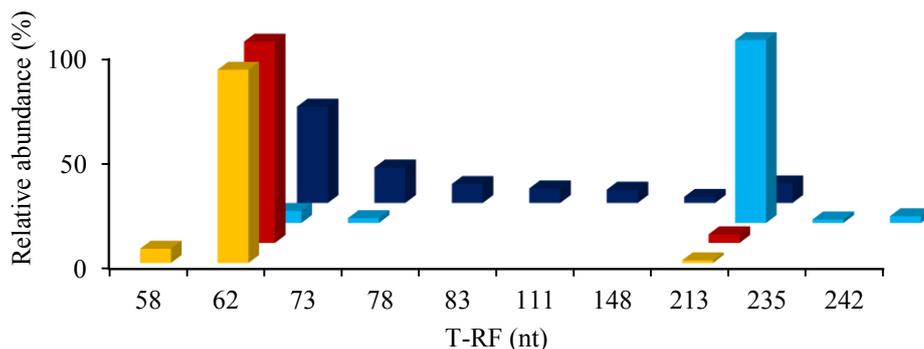


Figure 2 T-RFLP profiles of amplified archaeal 16S rRNA gene (digested with *HaeIII*) of filtrates from the monimolimnion water samples taken at 55 m (■) and 60 m (■), of the Guadiana pit lake sampled in October 2014, and the monimolimnion water samples taken at 15.2 m deep (■) and 35 m deep (■) sampled on March 2012.

Unlike the obtained bacterial clones, just one archaeal clone (H60A-9) was successfully obtained from the archaeal 16S rRNA clone library. This was 97% related to another clone (clone 38F-8) obtained from the monimolimnetic sediments of the pit lake Cueva de la Mora (Falagán et al. 2014). These clones are confined to the *Crenarchaeota* phylum, members of which are thought to be ubiquitous in aquatic and terrestrial environments (e.g. Abreu et al. 2001, Kemnitz et al. 2007)

Discussion

Prior to this study no indigenous bacteria or archaea had been identified in the monimolimnetic waters of the Guadiana pit lake, and archaea could only be detected in a similar pit lake of the IPB, the Cueva de la Mora pit lake (Falagán et al. 2014). The monimolimnetic waters of these lakes account 40 – 50% the lake volume. These waters are very rich in dissolved metals (e.g. Fe(II), Zn, Mn, As) and sulfate. Moreover, the monimolimnion of the Guadiana pit lake has extremely high CO₂ concentration, explained by water/rock interaction (carbonate dissolution) and by an inflow of ground water at 50 – 55 m deep (Sánchez-España et al. 2014, Boehrer et al. 2016).

In a recent study of these metal-mine pit lakes by Falagán et al. (2014), no DNA was obtained from a monimolimnetic water sample from the Guadiana pit lake. In the present work, although no isolates were obtained, archaeal and bacterial DNA was amplified from water samples taken from this anoxic layer. The T-RFLP profiles show that the bacterial communities were very similar in both water samples of the monimolimnion of the Guadiana pit lake, but they were different to that found in the upper layers of this lake (fig. 1; tab. 1). The archaeal community appeared to be very similar in waters from different depths within Guadiana, and were also similar to that found in the Cueva de la Mora monimolimnion (fig. 2).

Only a few bacteria were detected in the construction of the clone library; however, it is possible to have a good insight into the microbial composition of the monimolimnion. Clone HB55-11 was very closely related (identity of 95%) to *G. ferruginea*^T suggesting that it may not be the same species though it is likely to belong to the same genus. *Gallionella* is a group of iron-oxidizing bacteria firstly isolated from ferrous iron-containing fresh waters (Hallbeck and Pedersen 1990). *Gallionella* spp. form twisted stalks that are easily observed in water samples where inhabits, and use ferrous iron as energy source and carbon dioxide as carbon source (Hallbeck and Pedersen 1991), which both are found in high concentrations in the anoxic bottom layer of the Guadiana pit lake (Sánchez-España et al. 2014).

Clones H55B-2-1 and H55B-2-7 were found to be 99% related to uncultured clones obtained from deep-sea waters, and 98% related to *T. crunogena*^T. These clones (H55B-2-1 and H55B-2-7) were also 98% similar to an uncultured clone obtained from a marine shore sulfidic mine tailings dump (Chañaral Bay, Atacama Desert, northern Chile; Korehi et al. 2012). *T. crunogena* was firstly isolated from a hydrothermal vent at 2,600 m deep (Jannasch et al. 1985). This species can grow between pH 5.0 to 8.5 (Jannasch et al. 1985), whilst the monimolimnion of the Guadiana pit lake pH is 4.0 – 4.5. Given that the low pH at which *T. crunogena* can grow is higher than the pH of the monimolimnion, H55B-2-1 and H55B-2-7 could possibly be different species, but at least are distinct strains of the same species.

Thiomicrospira spp. are chemolithotrophic sulfur-oxidizing bacteria typically found in marine environments, *T. crunogena* uses reduced-sulfur compounds (i.e. sulfide, thiosulfate, and elemental sulfur) to obtain energy and carbon dioxide as carbon source (Jannasch et al. 1985).

Table 1. Identities of cloned genes obtained from the monimolimnion of the Guadiana pit lake and physiological characteristics of the closest described species. Genbank accession numbers are given in parenthesis.

Clone designation	Closest relative	% Identity (16S rRNA)	Fe/S transformations	pH range	Reference
H55B-11 (KX083694)	<i>Gallionella ferruginea</i> ^T	95	Fe ²⁺ -ox	5.0-6.5	Hallbeck and Pedersen 1990
H55B-27 (KX083695)	<i>Hydrogenovibrio marinus</i> ^T	92	S _{red} -OX	5.0-8.5	Nishihara et al. 1991
	<i>Thiomicrospira crunogena</i> ^T	91	S _{red} -OX	7.5-8.0	Jannasch et al. 1985
H55B-2-1 (KX083696)	<i>Thiomicrospira crunogena</i> ^T	98	S _{red} -OX	7.5-8.0	Jannasch et al. 1985
H55B-2-7 (KX083697)					
H60A-9 (KX083698)	Clone 38F-8	97			Falagán et al. 2014
	<i>Nitrososphaera viennensis</i> ^T	83		6-8.5	Stieglmeier et al. 2014

The other bacterial clone H55B-27 is distantly related to any described bacterial species (92% to *H. marinus*, and 91% to *T. crunogena*), and given this it is impossible to ascertain the metabolic function of this unknown bacterium.

Falagán et al. (2014) described the microbial composition of the upper layers of the Guadiana pit lake. The epilimnion of the Guadiana pit lake was dominated by heterotrophic bacteria belonging to the genera *Acidobacteriaceae*, *Acidocella* and *Acidisphaera*. The hypolimnion was the most microbially-diverse layer in the Guadiana pit lake. The dominant bacteria identified in this layer were very closely related to the iron-reducing moderate acidophile *Metallibacterium scheffleri*, and betaproteobacteria of the genus *Thiomonas* sp., which are sulfur-oxidizing bacteria typically found in acid mine drainage of pH 3 and above. Other bacteria able to catalyze biogeochemical transformations of iron and sulfur were also isolated from the hypolimnion of Guadiana. In contrast the majority of the bacterial clones from the monimolimnion are related to bacteria typically found in seawater. The salinity of the deep anoxic waters of this lake, mainly caused by SO₄²⁻, Fe²⁺ and Mg²⁺ (36 – 37 ‰) is very similar to the salinity of seawater, which is mainly caused Na⁺ and Cl⁻ (~ 35 ‰). Total dissolved solids in the monimolimnion of this metal-mine pit lakes increases with depth. The osmotic pressure under which the microorganisms might cope in this layer is therefore comparable to that found in seawater. Thus, the presence of microorganisms highly tolerant to the presence of salts is highly feasible in the monimolimnion of the Guadiana pit lake.

Three other meromictic mine pit lakes in the IPB have also been microbiologically characterized, Cueva de la Mora, the Concepción and the Nuestra Señora del Carmen pit lakes, the latter two of which experience periodic mixing. No bacteria were detected in the monimolimnion of the Cueva de la Mora pit lake Falagán et al. (2014), which is physico-chemically very similar to the Guadiana pit lake. *Nitrospira*, *Actinobacteria* and *Chloroflexi* were detected in the monimolimnion of the Nuestra Señora del Carmen while only *Betaproteobacteria* and *Actinobacteria* were detected in the monimolimnion of the Concepción pit lake (Santofimia et al. 2013).

Only one archaeal clone (H60A-9) was obtained from the 60 m sample, which was distantly related to all other classified archaeal species, though it is closely related to a clone obtained from a sediment sample collected from the Cueva de la Mora pit lake. Even though both clones are affiliated with the *Crenarchaeota* phylum, no affiliation to a genus or species level could be inferred. The major peaks present in the T-RFLP profiles of the archaeal 16S rRNA genes from this water layer have not been

identified. However, in a similar study carried out in the Cueva de la Mora pit lake, several clones obtained from water and sediment samples shared the same T-RF length (HaeIII digests) of 63 ± 2 nt. Those clones were grouped within the *Euryarchaeota* phylum, including clone 5A-1 which was obtained from a water sample from the monimolimnion in this lake (Falagán et al. 2014). Whether euryarchaeotes inhabit the monimolimnion of the Guadiana pit lake is uncertain as no clones with sequences related to any euryarchaeotal species were found. The similarity between Guadiana and Cueva de la Mora pit lakes could infer that this archaeal group may be also present in the Guadiana pit lake monimolimnion.

The presence of iron-oxidizing and reduced-sulfur compound oxidizing bacteria in the monimolimnion of the Guadiana pit lake suggests that transformations of both sulfur and iron are taking place within this zone, though electron acceptors other than oxygen (which is absent) would have to be used by these microorganisms. For example, *Sulfurihydrogenibium* (*S.*) *subterraneum* and *S. azorensis* use selenite or arsenate as electron acceptor (Takai et al. 2003, Aguiar et al. 2004, respectively), both of which are present in the monimolimnion of the Guadiana pit lake (Sánchez-España, personal communication). However, it is possible that enough oxygen could still be present in the monimolimnion at very low concentration difficult to detect, and acts as electron acceptor. The physico-chemical characteristics of pit lakes influence and are influenced by the microbial composition of their waters. The specific features of the metabolism of these bacteria and their role in the cycling of these two important elements clearly deserves further research.

This study highlights the scarce information existing of the bacterial and archaeal species inhabiting anoxic waters in metal-mine pit lakes in the IPB and the potential of these environments for discovering novel species of microorganisms, some of which could be potentially used in biotechnology.

Conclusions

The monimolimnion of the Guadiana pit lake is inhabited by bacteria that catalyze the oxidation of both ferrous iron and reduced-sulfur compounds, and therefore transformations of these elements are likely to be occurring in this anoxic zone of the pit lake.

The bacterial community of the Guadiana pit lake water column is divided in three different communities, corresponding to the physico-chemical stratification of the water column itself. The epilimnion is inhabited by phytoplankton and acidophilic heterotrophic iron-reducers, the hypolimnion by acidophilic iron- and sulfur-oxidizers, and the monimolimnion by neutrophilic iron-oxidizers and other bacteria related to that found in seawater. This is the first time that *Gallionella* has been reported in pit lakes of the IPB, as well as other species that are not typically found in acid mine waters, such as *Thiomicrospira* sp. The presence of salt-tolerant microorganisms in metal-mine pit lakes is probable and more effort needs to be done to isolate bacterial (and archaeal) species from the metal-mine lake waters as these are complex environments shown to be highly bacterially diverse.

While these findings shed new light on the complex microbial communities of metal-mine pit lakes, they also show that more research is required to understand fully how these both influence and are impacted by geochemistry of these environments.

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