

Geomicrobiology of meromictic, metal-mine pit lakes in the Iberian Pyrite Belt and biotechnological applications



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Tesis Doctoral 2015

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RESUMEN

Este trabajo aborda la caracterización microbiológica de dos lagos ácidos formados en antiguas explotaciones mineras de la Faja Pirítica Ibérica (Huelva, España), Cueva de la Mora y Guadiana (mina de Herrerías). Estos dos lagos se escogieron como modelo por sus especiales características hidrogeoquímicas y por su condición meromítica (estratificación permanente). Además de realizar una caracterización microbiológica detallada de ambos lagos, se describen nuevas especies bacterianas presentes en estas aguas ácidas con contenido muy alto de metales disueltos, y también se estudia la posibilidad de usar especies bacterianas presentes en estos ambientes extremos en procesos aplicados de biorremediación y biolixiviación.

Cueva de la Mora y Guadiana son dos ejemplos excepcionales de lagos meromíticos con marcados gradientes hidroquímicos y condiciones redox. La columna de agua de ambos lagos está dividida en dos capas, una capa superior (*mixolimnion*) oxigenada que sufre periodos de estratificación durante los meses más calurosos y una capa inferior anóxica (*monimolimnion*) permanentemente aislada de la atmósfera oxidante. Estas dos capas están separadas por una zona de transición (*quimioclina/redoxclina*), que presenta fuertes variaciones verticales de pH, potencial redox y oxígeno disuelto en un espesor de unos pocos metros. Ambos lagos se caracterizan por tener altas concentraciones de metales disueltos (p. ej. Fe, Al, Mn, Cu, Zn, Co, Ni) en toda la columna de agua, y escasas concentraciones de nutrientes (P y N). El metal predominante en ambos lagos es el hierro que está presente como hierro férrico (Fe(III)) en la capa superior y como hierro ferroso (Fe(II)) en la capa inferior.

Gracias al uso de técnicas basadas en biología celular y molecular se ha podido determinar la existencia de tres hábitats distintos en la columna de agua de los lagos Cueva de la Mora y Guadiana poblados por comunidades microbiológicas diferentes. Y además se han observado otras comunidades microbianas características de los sedimentos. La columna de agua de ambos lagos está poblada por bacterias acidófilas (p. ej. *Acidithiobacillus ferrooxidans*, *Acidithiobacillus ferrivorans*, *Leptospirillum ferrooxidans*, *Acidicapsa* sp.) que se distribuyen de forma diferente entre las distintas capas. Mientras que la zona óxica está principalmente poblada por organismos fotosintéticos (p. ej. *Pseudococcomyxa simplex*) y bacterias reductoras de hierro

(*Acidicapsa* sp.), la capa de transición presenta mayor diversidad microbiana. En ambos lagos, esta zona de transición está fuertemente condicionada por los ciclos bioquímicos del hierro y del azufre, que se manifiestan con claridad y ejercen un importante control sobre la hidroquímica del lago. Sin embargo, el monimolimnion de ambos lagos es la zona que muestra menor presencia y diversidad bacteriana, hasta el punto de que no se ha podido detectar ninguna especie bacteriana en estas aguas. La distribución vertical de los nutrientes (especialmente el fósforo) parece que juega un papel muy importante en la ecología microbiana de estos lagos determinando la distribución del fitoplancton en la columna de agua que a su vez actúa como fuente de carbono para las capas de agua inferiores. Los sedimentos recogidos de la zona óxica y de la zona anóxica se caracterizan por presentar comunidades microbianas diferentes a las observadas en la columna de agua, siendo también distintas entre ellos. Los sedimentos de la zona óxica son más diversos en cuanto a metabolismos bacterianos dado que aparecen bacterias tanto aeróbicas (p. ej. bacterias oxidadoras de hierro) como anaeróbicas (p. ej. bacterias sulfato-reductoras) mientras que los sedimentos de la zona anóxica están poblados por metabolismos bacterianos anaeróbicos (p. ej. sulfato-reducción). Además, se ha investigado la posible presencia de arqueas en aguas de ambos lagos y en sedimentos. Este grupo de procariotas se ha detectado en aguas del monimolimnion habiéndose observado también en sedimentos tanto de la zona óxica como de la anóxica. Entre las arqueas detectadas, se han encontrado ciertos grupos típicos de ambientes con pH más elevado (p. ej. arqueas metanógenas) incluyendo un filo (*Thaumarchaeota*) que incluye especies capaces de oxidar amonio.

Durante esta investigación, se aislaron un gran número de bacterias en los lagos Cueva de la Mora (columna de agua y sedimentos) y Guadiana (columna de agua). Aunque se encontraron especies de bacterias acidófilas conocidas mundialmente, incluyendo *Acidithiobacillus ferrooxidans* y *Leptospirillum ferrooxidans*, también se han encontrado otras que han resultado corresponder a nuevas especies, e incluso, a géneros bacterianos nuevos que no han sido descritos hasta la fecha (p. ej. "*Acidicapsa ferrireducens*", *Acidibacter ferrireducens*, "*Clostridium acididurans*". De entre todas estas, se eligieron seis para su caracterización fisiológica y se estudió su crecimiento en presencia de algunos metales que están típicamente presentes en estos lagos ácidos (p. ej. Cu, Al, Mn). Como resultado, se proponen cuatro nuevas especies ("*Acidicapsa ferrireducens*", "*Acidicapsa acidophila*", "*Granulicella acidophila*" y *Acidibacter ferrireducens*) y un género nuevo (*Acidibacter* sp.) de bacterias acidófilas, así como una especie de bacteria acido-tolerante ("*Clostridium acididurans*"). Tres de estas nuevas

especies (*Acidicapsa ferrireducens*, *Acidicapsa acidophila* y *Acidibacter ferrireducens*) son capaces de realizar la reducción disimilatoria de hierro (III) en condiciones microaeróbicas y sólo una de ellas también en condiciones anaeróbicas (*Acidibacter ferrireducens*). Mientras que una de estas nuevas especies sólo es capaz de crecer en condiciones aeróbicas (*Granulicella acidophila*) otra sólo crece bajo estrictas condiciones de anaerobiosis (*Clostridium acididurans*). Los estudios de crecimiento en presencia metales se complementaron con microscopía electrónica en busca de evidencias de acumulación intra- o extracelular de alguno de estos metales. Estos estudios han mostrado altos niveles de tolerancia de la gran mayoría de las bacterias estudiadas a metales o metaloides tales como el Mn(II), el Al, el As(V) o el Mg(II). No así para otros metales como el Cu(II) o el Fe(II), para la que estas bacterias presentaron baja tolerancia (excepto *Acidibacter ferrireducens*). Los elevados contenidos de este último metal en el monimolimnion de ambos lagos (donde se han medido concentraciones superiores a 100 mM de Fe(II)) podrían estar condicionando la presencia y desarrollo de comunidades bacterianas en estas zonas profundas de quimismo tan extremo. Sólo una de las bacterias aisladas presentaba precipitados extracelulares de hierro.

En relación al ciclo del nitrógeno, cabe destacar que este elemento es un nutriente esencial para el desarrollo de microorganismos, incluyendo el fitoplancton y por tanto su presencia y biodisponibilidad condiciona, junto con el fósforo, la producción primaria en masas de agua. El nitrógeno está disuelto en forma de amonio (NH_4^+) y nitrato (NO_3^-) en la columna de agua de ambos lagos, Cueva de la Mora y Guadiana. Mientras la concentración de amonio incrementa en profundidad (zona anóxica, monimolimnion), el nitrato se mantiene a baja concentración en toda la columna de agua, aunque es algo más abundante en la zona oxigenada superficial (mixolimnion). Se ha estudiado el papel del nitrato y del amonio en la columna de agua de ambos lagos y su posible relación con los microorganismos que habitan en ella mediante cultivos celulares para la determinación de los metabolismos bacterianos de desnitrificación (reducción de nitrato acoplada a la oxidación de hierro) y nitrificación (oxidación de amonio a nitrato en presencia de oxígeno). Estos experimentos se llevaron a cabo en macrocosmos, en condiciones aeróbicas y anaeróbicas, durante los cuales se monitorizaron las concentraciones de hierro, nitrato, amonio y carbono orgánico disuelto. Además, para la detección de nitrificación, los macrocosmos se inocularon con el isótopo de nitrógeno, ^{15}N , en forma de $^{15}\text{NH}_4\text{Cl}$, monitorizando la presencia de éste en el producto final del proceso. La principal conclusión de estos trabajos es que tanto nitrificación como

denitrificación son aparentemente inviables en la columna de agua de ambos lagos. Sin embargo, la detección de arqueas oxidadoras de amonio en sedimentos de Cueva de la Mora deja abierta la posibilidad de la existencia de este metabolismo a pesar del bajo pH predominante en estos ambientes, e invita a efectuar estudios de detalle en el futuro.

Tras la caracterización microbiológica de ambos lagos, se realizaron estudios de detalle desde un punto de vista aplicado a la remediación de problemas medioambientales asociados al drenaje ácido de mina. El aluminio es uno de los metales más abundantes en la columna de agua tanto de Cueva de la Mora como de Guadiana. Este catión no tiene papel biológico conocido y además es tóxico para muchos organismos. En este trabajo se ha estudiado la posibilidad de movilizar el aluminio disuelto en aguas ácidas favoreciendo su precipitación mediante el uso de un biorreactor que contenía una población mixta de bacterias sulfato-reductoras acidófilas. Durante más de dos meses, se mantuvo el reactor en flujo continuo siendo alimentado con una solución con alta concentración de aluminio que simulaba las aguas ácidas de mina. Estos ensayos indican que el aluminio, que precipita en forma de oxi-hidroxisulfatos como la hidrobasaluminita y la felsöbanyaita a $\text{pH} > 4.5$, puede ser removilizado de la fase acuosa gracias a la actividad de bacterias sulfato-reductoras. La oxidación anaeróbica de sustratos orgánicos por medio de sulfato es una reacción que requiere protones, las bacterias sulfato-reductoras usan los protones procedentes de la formación de oxi-hidroxisulfatos de aluminio, lo que hace que el pH en el biorreactor se mantenga por encima de 4.5, favoreciendo así la precipitación del aluminio. Existen evidencias que sugieren que este proceso se está dando de forma natural en la quimioclina del lago Cueva de la Mora, lo que sugiere que la potenciación de esta actividad sulfato-reductora en estos lagos ácidos (p. ej. mediante la adición de sustratos orgánicos apropiados para estas bacterias) podría servir como posible vía para reducir la contaminación de sus aguas.

Por otro lado, se han llevado a cabo varios experimentos para optimizar la disolución de calcopirita (CuFeS_2). Este mineral es uno de los más abundantes minerales de cobre en la litosfera y está presente en la mena mineral de muchas de las minas de la FPI (p. ej. es el segundo sulfuro más abundante en la mina de Cueva de la Mora), además de constituir una fuente de contaminación adicional a la pirita. La calcopirita es recalcitrante con respecto del biolixiviado y sólo un 30% del cobre se ha conseguido extraer por métodos convencionales. Los experimentos de lixiviación se realizaron tanto en condiciones abióticas como en presencia de bacterias, en

condiciones tanto anaeróbicas como aeróbicas, y en presencia de hierro disuelto. Los mejores resultados se obtuvieron en presencia de bacterias oxidadoras de hierro y de bacterias oxidadoras de azufre, siendo el hierro (III) regenerado biológicamente. Se consiguió lixiviar un 50% del cobre presente en el concentrado de calcopirita utilizado. En ausencia de hierro la lixiviación de la calcopirita se vio detenida al cabo de unas horas, y aunque se pudo reanudar añadiendo hierro (III), el biolixiviado se detuvo al cabo de dos semanas. Además, los resultados de estos experimentos muestran que, a diferencia de lo que se suele considerar, la biolixiviación de la calcopirita no se ve favorecida al eliminar la película de azufre nativo que se forma sobre las superficies reactivas como resultado de la oxidación de la calcopirita. Mediante un ajustado control sobre el potencial redox de la solución, se estimuló la regeneración biológica de hierro (III) llegando a extraer más de la mitad del cobre presente en este concentrado de calcopirita. El método aún puede optimizarse y es necesario que en el futuro se realicen más experimentos para determinar el papel que juega el hierro disuelto y cómo se pueden mejorar los resultados obtenidos en este trabajo.

En resumen, este estudio proporciona: (i) una completa caracterización microbiológica de dos lagos ácidos formados en antiguas explotaciones mineras de la Faja Pirítica Ibérica (Huelva, España), (ii) la descripción de nuevas especies bacterianas aisladas de muestras de agua de estos lagos, (iii) la respuesta de estas nuevas especies bacterianas a la presencia de altas concentraciones de metales típicamente disueltos en lagos ácidos; (iv) la influencia de la distribución de nutrientes sobre la comunidad microbiana en estos ambientes; (v) posibles vías de biorremediación de aguas ácidas de mina y residuos mineros mediante la precipitación de metales (aluminio) y/o (vi) la optimización del proceso de lixiviado de la calcopirita, uno de los sulfuros de mena más abundantes en las minas de la Faja Pirítica Ibérica y presente en los residuos mineros.

ABSTRACT

This work is based on the microbiological characterization of two acid mine pit lakes formed in two abandoned mine sites in the Iberian Pyrite Belt (IPB), Cueva de la Mora and Guadiana (Herrerías mine). These two lakes were chosen among many others because of their outstanding hydrogeochemical features and their stratification pattern, which in both lakes is permanent (meromictic). The microbial characterization of both lakes is presented in this thesis. In addition, new bacterial species isolated from these lakes are described. Finally, the possible application of typical species inhabiting these acid environments on bioremediation and bioleaching is also described.

Cueva de la Mora and Guadiana pit lakes are exceptional examples of meromictic stratification with vertical gradients of water chemistry and redox conditions. The lakes water columns are divided in two main layers, the upper oxic layer (mixolimnion) which experiences periods of stratification during the warmer months, and the bottom anoxic layer (monimolimnion) which is completely isolated from the atmosphere. The transitional layer (which separates the upper and the bottom layers) is very narrow and presents sharp gradients of pH, redox potential, and dissolved oxygen content. Both lakes are characterized by high metal concentrations (e.g. Fe, Al, Mn, Cu, Zn, Co, Ni) in the water column and by relatively low nutrients (P and N) concentrations. Iron is the dominant metal in the lake waters, being mainly ferric ion in the mixolimnion, and ferrous ion in the monimolimnion.

A combined cultivation-based and culture-independent study of both lakes revealed the presence of different microbial environments within the lake waters, but also within the lake sediments. The lake waters are populated by acidophilic bacteria (*Acidithiobacillus ferrooxidans*, *Acidithiobacillus ferrivorans*, *Leptospirillum ferrooxidans*, *Acidicapsa* sp.) that are distributed differently among the different layers. Phototrophic microorganisms (e.g. *Pseudococcomyxa simplex*) and ferric iron reducers are the two dominant microbial groups in the upper layer. The transition layer shows the highest bacterial diversity, moreover the iron and sulphur biogeochemical cycles determine the water chemistry in this depth. No bacteria were detected in the monimolimnetic waters. Nutrients distribution has a major role in the lakes microbial distribution. Specially phosphorus that determines the phytoplanktonic growth in the lake waters and its

distribution. These photosynthetic microorganisms provide organic carbon for the microbial community present in the underlying waters. Sediment samples from the oxic and the anoxic zones are populated by different microbial communities that are also different from those found in the water column of the lake. Sediments from the oxic zone are inhabited by aerobic (e.g. iron oxidizers) and anaerobic (e.g. sulphate-reducers) bacteria whilst sediments from the anoxic zone are exclusively populated by diverse anaerobic bacteria (e.g. sulphate-reducing bacteria). In addition, archaea were also detected in the lake waters and sediments. Some archaeal groups typically found in less acidic environments (e.g. methanogens) were detected in the lake sediments including a recently described phylum of ammonia oxidizing archaea (*Thaumarchaeota*).

Among the bacterial species isolated from both pit lakes, Cueva de la Mora (water and sediment samples) and Guadiana (water samples), some of them were well-known acidophiles, including *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*. However, some other corresponded to new bacterial species and some also to new genera (e.g. "*Acidicapsa ferrireducens*", *Acidibacter ferrireducens*, "*Clostridium acididurans*"). Six of these new strains were chosen for a detailed physiological characterization. These strains were grown under the presence of metals typically found at high concentrations in acidic mine pit lakes (e.g. Cu, Al, Mn), and bacterial cells were studied under the electron microscope in order to find evidences of intra- or extracellular metal accumulation. Four new species ("*Acidicapsa ferrireducens*", "*Acidicapsa acidophila*", "*Granulicella acidophila*" and *Acidibacter ferrireducens*) and a new genus (*Acidibacter* sp.) of acidophilic bacteria, and a new acid-tolerant species ("*Clostridium acididurans*") are proposed in this work. Three of these new species ("*Acidicapsa ferrireducens*", "*Acidicapsa acidophila*" and *Acidibacter ferrireducens*) are able to perform the dissimilatory reduction of ferric iron under microaerobic conditions and one of them (*Acidibacter ferrireducens*) also under anaerobic conditions. Among these new bacteria, one of them is a strict aerobe ("*Granulicella acidophila*") and another one is a strict anaerobe ("*Clostridium acididurans*"). The majority of the isolates are highly tolerant to different metals or metalloids such as Mn(II), Al, As(V) and Mg(II). However, they are not very tolerant to Cu(II) or Fe(II) (except for *Acidibacter ferrireducens*). The high concentration of ferrous iron in the monimolimnetic waters (>100 mM Fe(II)) could be determining the development of bacterial communities in this anoxic layer. Among all the isolates studied only one presented evidences of extracellular iron precipitation.

With respect to the cycling of nitrogen, it must be considered that this metal is an essential nutrient for microbial growth (including phytoplankton), and, therefore, controls the primary production (along with phosphate) in water masses. Dissolved inorganic nitrogen is mainly present as ammonium (NH_4^+) and nitrate (NO_3^-) in the water column of both pit lakes, Cueva de la Mora and Guadiana. Ammonium concentration increases in depth while nitrate is slightly more abundant in the upper oxic layer. The microbial influence on the destination and distribution of nitrate and ammonium in the water column in both lakes has been evaluated. The possible existence of bacterial denitrification (nitrate reduction coupled to iron oxidation) and nitrification (ammonium oxidation in the presence of oxygen) in the lake waters was determined. Several macrocosms were set under aerobic and anaerobic conditions. The concentrations of iron, nitrate, ammonium, and dissolved organic carbon were monitored over both nitrification and denitrification experiments. The detection of nitrification was also studied by using a nitrogen isotope by inoculating the macrocosms with $^{15}\text{NH}_4\text{Cl}$ and monitoring the presence of ^{15}N in the last product of the process. The main conclusion of these experiments is that both processes (nitrification and denitrification) are not viable in the lake waters. However, the detection of an ammonia oxidizing archaea phylum in a sediment sample of the Cueva de la Mora pit lake suggests that ammonia oxidation may be occurring in the lake sediments.

After the microbial characterization of both lakes, the investigations were focused on the potential application of geomicrobial (metal/microorganism) interactions for biotechnological use. Specifically, the possible use of some bacterial metabolisms (e.g. sulphate-reduction, iron oxidation) for bioremediation and bioleaching were evaluated. As regards the bioremediation options, aluminium is one of the most abundant elements in the Cueva de la Mora and Guadiana pit lake, and has no known biological role being toxic for many organisms. In this research, the possibility of mobilizing dissolved aluminium in acid waters was studied using a sulphate-reducing bacteria consortium in a continuous-flow bioreactor. During two months, the reactor was fed with a high aluminium concentration liquor simulating acid mine waters. These experiments indicated that aluminium, which precipitates as oxi-hydroxysulphates (e.g. hydrobasaluminite and felsöbanyaite at $\text{pH} > 4.5$), can be mobilized by the sulphate-reducing bacterial activity. The anaerobic oxidation of organic carbon by sulphate requires protons, the sulphate-reducing bacteria populating the reactor use the protons produced during the formation of aluminium oxi-hydroxysulphates keeping the reactor liquor at $\text{pH} > 4.5$ and favouring the precipitation of aluminium. There are evidences

showing that this process occurs naturally in the chemocline of Cueva de la Mora. These findings suggest that favouring the sulphate-reducing activity in the lake waters should be considered and studied as a mechanism to reduce the lake water contamination.

Finally, several experiments in order to optimize the chalcopyrite (CuFeS_2) leaching were carried out. Chalcopyrite is one of the most abundant copper minerals in the lithosphere. This mineral is also a common constituent in the mineral ore of many mines in the Iberian Pyrite Belt being the second most abundant mineral in Cueva de la Mora mine ore and also represents an additional source of metal pollution. Chalcopyrite is recalcitrant to bioleaching and only 20-30% of its copper content is leached by conventional oxidative bioleaching. Several experiments were carried out abiotically and in the presence of bacteria, under anaerobic and aerobic conditions, and in the presence of dissolved iron. The leaching of the chalcopyrite concentrate was more effective in the presence of iron- and sulphur-oxidizing bacteria where ferric iron was regenerated biologically. Around 50% of the copper was extracted from the chalcopyrite concentrated utilized. In the absence of iron, the chalcopyrite leaching came to a halt; adding ferric iron made it to start again, although the process finally stopped after two weeks. The removal of sulphur did not result in enhanced bioleaching of the concentrate. The biological regeneration of ferric iron and the control of the redox potential helped to extract more than the 50% of the copper present in the chalcopyrite concentrated. More experiments should be done to determine the role of dissolved iron in chalcopyrite bioleaching and to optimize this process.

Summarizing, this study provides: (i) the microbial characterization of two acid meromictic, metal-mine pit lakes in the Iberian Pyrite Belt; (ii) the characterization of new bacterial species isolated from these two lakes; (iii) the interactions of these new bacterial isolates with dissolved metals typically found in acidic mine waters; (iv) a study of the influence of nutrient distribution in the microbial community; (v) an evaluation of the potential application of bacterial metabolisms (sulphate-reduction) to induce the precipitation of dissolved metals (aluminium) in acid waters, and (vi) an approach for optimizing the bioleaching of chalcopyrite in biomining processes.

INDEX

1	INTRODUCTION AND OBJECTIVES	1
1.1	THE IBERIAN PYRITE BELT	3
1.1.1	HISTORICAL BACKGROUND	3
1.1.2	GEOLOGICAL FRAMEWORK OF THE IPB	5
1.2	BASIC CONCEPTS OF ACID MINE DRAINAGE CHEMISTRY	9
1.3	PHYSICO-CHEMICAL CHARACTERISTICS OF ACID PIT LAKES	11
1.4	MICROBIOLOGY IN ACIDIC ENVIRONMENTS	19
1.5	MICROBIOLOGY OF ACID PIT LAKES	23
1.6	MICROBIOLOGY IN THE IPB	25
1.6.1	MICROBIOLOGY OF THE TINTO RIVER	25
1.6.2	MICROBIOLOGY OF IBERIAN PIT LAKES	27
1.7	GENERAL CONCEPTS OF BIOMINING	28
1.8	OBJECTIVES	31
2	PHYSICO-CHEMICAL CHARACTERIZATION OF CUEVA DE LA MORA AND GUADIANA PIT LAKES	33
2.1	INTRODUCTION	36
2.2	MATERIALS AND METHODS	37
2.2.1	STUDY SITES AND SAMPLING TECHNIQUES	37
2.2.2	LABORATORY-BASED CHEMICAL ANALYSES	40
2.3	RESULTS	40
2.3.1	WATER COLUMN CHEMISTRY	41
2.3.2	SEDIMENT CHEMISTRY	46
2.4	DISCUSSION	47
2.5	CONCLUSIONS	51

3	MICROBIOLOGY OF CUEVA DE LA MORA AND GUADIANA PIT LAKES	53
3.1	INTRODUCTION	56
3.2	MATERIALS AND METHODS	57
3.2.1	SAMPLING TECHNIQUES AND SAMPLING DATES	57
3.2.2	PIGMENT ANALYSES	58
3.2.3	DETERMINATION OF VIABLE COUNTS OF MICROORGANISMS AND ISOLATION OF ACIDOPHILES	58
3.2.4	BIOMOLECULAR ANALYSES	60
3.3	RESULTS	63
3.3.1	WATER MICROBIOLOGY	63
3.3.1.1	PHOTOTROPHIC COMMUNITY	63
3.3.1.2	PROKARYOTIC COMPOSITION	69
3.3.1.2.1	CUEVA DE LA MORA	70
3.3.1.2.2	GUADIANA PIT LAKE	75
3.3.2	SEDIMENT MICROBIOLOGY: CUEVA DE LA MORA	76
3.3.3	IDENTIFICATION AND CHARACTERISTICS OF ACIDOPHILIC BACTERIA ISOLATED FROM THE CUEVA DE LA MORA AND GUADIANA PIT LAKES	78
3.4	DISCUSSION	78
3.5	CONCLUSIONS	87
3.6	APPENDIX	89
4	NEW BACTERIAL ISOLATES AND THEIR RESISTANCE TO DISSOLVED METALS	93
4.1	INTRODUCTION	96
4.2	MATERIALS AND METHODS	99
4.2.1	CULTIVATION OF DIFFERENT ACIDOPHILIC HETEROTROPHS FROM GUADIANA AND CUEVA DE LA MORA PIT LAKES	99
4.2.2	CHEMOTAXONOMIC ANALYSIS	100

4.2.3 PIGMENT ANALYSES	100
4.2.4 GRAM STAINING TEST	101
4.2.5 MOTILITY SCREENING	101
4.2.6 PIGMENT ANALYSES	101
4.2.7 SCREENING FOR CHEMOLITHOTROPHIC AND CHEMOLITHO- HETEROTROPHIC GROWTH	101
4.2.8 GROWTH ON ORGANIC SUBSTRATES	102
4.2.9 EFFECTS OF PH AND TEMPERATURE ON THE GROWTH RATE OF DIFFERENT HETEROTROPHS FROM THE PIT LAKES	102
4.2.10 IRON REDUCTION AND TESTING FOR GROWTH IN ANAEROBIC CONDITIONS	103
4.2.11 SENSITIVITY TO TRANSITION METALS, ALUMINIUM AND SALT	104
4.3 RESULTS	105
4.3.1 MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERIZATION	105
4.3.2 CHEMOTAXONOMIC AND PHYLOGENETIC ANALYSIS	111
4.3.3 IRON REDUCTION AND TESTING FOR GROWTH IN ANAEROBIC CONDITIONS	115
4.3.4 SENSITIVITY TO TRANSITION METALS, ALUMINIUM AND SALT	116
4.4 DISCUSSION	119
4.4.1 ISOLATES MCF9, MCF10 AND MCF14	119
4.4.2 ISOLATE MCF40	120
4.4.3 ISOLATE MCF85	120
4.4.4 ISOLATE MCF105	120
4.4.5 METAL TOLERANCE	121
4.5 CONCLUSIONS	122
4.6 APPENDIX	124

5	MICROBIALY MEDIATED NITROGEN CYCLING IN ACID MINE PIT LAKES OF THE IBERIAN PYRITE BELT	125
5.1	INTRODUCTION	128
5.2	MATERIALS AND METHODS	131
5.2.1	SAMPLING	131
5.2.2	DENITRIFICATION COUPLED TO IRON OXIDATION SET UP	131
5.2.3	NITRIFICATION EXPERIMENTS SET UP	133
5.2.4	LABORATORY-BASED CHEMICAL ANALYSES	134
5.3	RESULTS	134
5.3.1	DENITRIFICATION	135
5.3.2	NITRIFICATION	139
5.4	DISCUSSION	140
5.5	CONCLUSIONS	142
6	BIOLOGICALLY-INDUCED PRECIPITATION OF ALUMINIUM IN SYNTHETIC ACID MINE WATER	145
6.1	INTRODUCTION	148
6.2	MATERIALS AND METHODS	151
6.2.1	BIOREACTOR SETTINGS	151
6.2.2	ANALYTICAL METHODS	154
6.2.3	BIOMOLECULAR ANALYSES	155
6.3	RESULTS	156
6.3.1	BIOREACTOR	156
6.3.2	ANALYSES OF PRECIPITATES	158
6.3.3	BACTERIAL BIOREACTOR POPULATION	160
6.4	DISCUSSION	162
6.5	CONCLUSIONS	166

7	IMPLICATIONS OF BIOLEACHING: ABIOTIC AND MICROBIAL DISSOLUTION OF CHALCOPYRITE	169
7.1	INTRODUCTION	172
7.2	MATERIALS AND METHODS	173
7.2.1	MINERAL CONCENTRATE	173
7.2.2	MICROORGANISMS	174
7.2.3	LEACHING OF CHALCOPYRITE IN A pH- AND TEMPERATURE-CONTROLLED REACTOR VESSEL	174
7.2.4	FERRIC IRON LEACHING OF A COPPER (I) SULPHIDE (CHALCOCITE/DJURLEITE) MINERAL SAMPLE AT LOW REDOX POTENTIALS	175
7.2.5	ACID LEACHING OF A COPPER (I) SULPHIDE (CHALCOCITE/DJURLEITE) MINERAL SAMPLE IN THE ABSENCE OF FERROUS IRON	176
7.2.6	CHEMICAL ANALYTICAL METHODS	176
7.2.7	MINERALOGICAL ANALYSIS	177
7.3	RESULTS	177
7.3.1	ABIOTIC ANAEROBIC DISSOLUTION OF CHALCOPYRITE BY FERRIC IRON UNDER CONTROLLED REDOX POTENTIAL	177
7.3.2	MICROBIALY-CATALYSED DISSOLUTION OF CHALCOPYRITE AT LOW REDOX POTENTIALS	179
7.3.3	BIOLEACHING OF CHALCOPYRITE UNDER LOW REDOX POTENTIALS IN THE PRESENCE OF ADDITIONAL FERROUS SULPHATE	180
7.3.4	MINERALOGICAL CHANGES IDENTIFIED IN THE BIOLEACHED CHALCOPYRITE CONCENTRATE	181
7.3.5	ABIOTIC LEACHING OF CHALCOCITE/DJURLEITE	181
7.4	DISCUSSION	183
7.5	CONCLUSIONS	186
8	GENERAL CONCLUSIONS	189
9	BIBLIOGRAPHY	195

**INTRODUCTION AND
OBJECTIVES**

1

1.1 THE IBERIAN PYRITE BELT	3
1.1.1 HISTORICAL BACKGROUND	3
1.1.2 GEOLOGICAL FRAMEWORK OF THE IPB	5
1.2 BASIC CONCEPTS OF ACID MINE DRAINAGE CHEMISTRY	9
1.3 PHYSICO-CHEMICAL CHARACTERISTICS OF ACID PIT LAKES	11
1.4 MICROBIOLOGY IN ACIDIC ENVIRONMENTS	19
1.5 MICROBIOLOGY OF ACIDIC PIT LAKES	23
1.6 MICROBIOLOGY IN THE IPB.....	25
1.6.1 MICROBIOLOGY OF THE TINTO RIVER	25
1.6.2 MICROBIOLOGY OF IBERIAN PIT LAKES.....	27
1.7 GENERAL CONCEPTS OF BIOMINING	28
1.8 OBJECTIVES	31

1.1 THE IBERIAN PYRITE BELT

The Iberian Pyrite Belt (IPB) is located in the south-west of the Iberian Peninsula and it extends from the south of Lisbon to the west of Seville. This famous mining district contains many massive sulphide deposits of Palaeozoic (Carboniferous) age classified as giant and supergiant (Sáez et al. 1999), being one of the largest massive sulphide provinces on Earth (Leistel et al. 1998). Several authors have estimated that the total ore reserves exceed 1500 Mt (Leistel et al. 1998, Sáez et al. 1999). These massive sulphide bodies consist of pyrite, associated with sphalerite, galena, chalcopyrite, and many other minor phases (Velasco et al. 1998, Sáez et al. 1999).

1.1.1 HISTORICAL BACKGROUND

The IPB has been mined for centuries. There are archaeological evidences of mine activity since the metal ages. During Tartessian and Roman times, the IPB mines were exploited to obtain gold, silver and copper (Pinedo Vara 1963). The Roman times were an intense period of exploitation, but mining activity stopped during the Middle Ages and resumed two centuries ago. The most intense mining activity took place in the Modern age, especially between 1880 and 1980. In 1990s, the drastic fall in the price of metals and the rise of costs in the metallurgical industry caused most mines in the region to be abandoned. At present, there are only two active mines in the IPB (Aguas Teñidas in Huelva, and Las Cruces in Seville), though there are several projects of mine re-opening in the area.

When mining activity started in the 19th century, the works were firstly focused on underground activity, although during the second part of the century, open mining activity flourished combined with underground works to exploit most of the minerals that could not be extracted exclusively by underground mining (Pinedo Vara 1963). The pyrite ores mined in the IPB were chiefly used for sulphur extraction for the chemical industry (e.g. sulphuric acid production). In 1858, the development of new processes capable of recovering metals (Au, Ag, Cu, Pb, Zn) from the ashes obtained from pyrite roasting helped to increase the economic value of these mines so that the mining activity increased again. At the end of the 19th century, the price of Iberian pyrites decreased due to several factors, including: (i) the development of a new method of sulphuric acid production from the oil industry, (ii) a drop of the copper price in the international metal

market, and (iii) the discovery and opening of some big mines in Chile, USA and South Africa.

During the 20th century, the price of copper was highly variable owing to the contrasting effects of the First and Second World Wars (which increased the demand of metals) and to the opening of new mines around the world (which increased the metal reserves and caused subsequent decreases in the price of metals). The sulphur prices followed a similar tendency. In the 1950s, this element increased due to the scarcity of sulphur around the world and because of the Korean War. However, this situation did not last for long and sulphur soon started to be obtained from other industrial processes available at lower costs. In addition, the environmental regulation of metal mining became stricter, discouraging companies from starting new mining projects. On the other hand, copper demand increased in the 1970s and until 1982, when the use of other materials (aluminium, optical fibre, recycled compounds) became more popular (Carrasco 2000).

Only in the Spanish part of the IPB, more than 30 pit lakes formed as a result of the abandonment and subsequent flooding of the former open pits (Sánchez-España et al. 2008, López-Pamo et al. 2009). The long and intensive mining activity has left a deeply modified landscape and a legacy of many different contamination sources in watercourses in the southwest of the Iberian Peninsula (Sánchez-España et al. 2005). These sources include spoils heaps, waste rock piles, tailings, seepage emerging from shafts and mine portals, and outflows from pit lakes. The two lakes this work is focused are: the Cueva de la Mora pit lake, which is located in the mine with the same name, and the Guadiana pit lake, which is located in Herrerías mine. Cueva de la Mora mine was first opened by a Portuguese mining company in 1875, and closed in 1930 after the world copper crisis and the political situation of Spain. In the same year, the void was completely flooded. When the mine was re-opened, the void was emptied, until 1971 when the mine was finally abandoned. In the Herrerías mine, at the Guadiana site, mining works first started in 1895 by a British company, but in 1941 the open mine works stopped and the void was flooded. During the 1980s, the exploitation of the mine was exclusively underground so the water was pumped out until 1995 when the pumping activity stopped and the void started to be flooded again (López-Pamo et al. 2009, Sánchez-España et al. 2014a, 2014b).

1.1.2 GEOLOGICAL FRAMEWORK OF THE IPB

The Iberian Pyrite Belt forms an arch of 240 km length and 35 km width between Seville and Grândola (South of Lisbon, Portugal) (Leistel et al. 1998) (Fig. 1.1). From a geological perspective, the IPB belongs to the South Portuguese Zone (Julivert 1974). The stratigraphic record of the IPB consists of Late Devonian-Early Carboniferous materials covered by Tertiary and Quaternary sediments (including fluvial sediments). The stratigraphic sequence is formed by three main lithographic units (Sáez et al. 1996, Leistel et al. 1998, Ruiz de Almodóvar et al. 1998, Tornos 2006): the bottom unit is a 2,000 m-thick layer of phyllites and quartzites, the Phyllite-Quartzite Group (PQ Group); this unit is followed by the Volcano-Sedimentary Complex (VSC), made of volcanic and sedimentary rocks, and hosting the massive sulphide mineralizations; and finally, the top sequence consists in the Culm Group, a 1,000 m-thick unit composed of shales and greywakes.

The thickness of the VSC is not homogeneous, and may locally range from 0 to 1,300 m (Tornos 2006). The relative proportion of volcanic and sedimentary rocks is also variable. The northern part consist in a volcanic sequence with some layers of slates, while the southern part is characterized by the abundance of siliciclastic sedimentary rocks with a continental influence associated with the volcanic rocks. The most abundant rocks of the VSC are acid volcanic rocks, including lava flows and tuffs of rhyolitic to dacitic composition (Sáez et al. 1996, Tornos 1998, 2006, Sánchez-España 2000). However, the volcanic lithology also include intermediate and basic (basaltic) volcanic rocks. Hydrothermal systems are usually located in areas of subaqueous volcanism (Lydon 1984, 1988, 1996, Large 1992). The volcanogenic massive sulphides (VMS) of the IPB were formed by the interaction of hydrothermal mineralizing fluids with the ocean floor (Sáez et al. 1996, Leistel et al. 1998, Ruiz de Almodóvar et al. 1998, Velasco et al. 1998, Tornos 1998, 2008, Sánchez-España et al. 2000, 2003).

The IPB hosts more than 1,600 Mt of massive sulphides and about 250 Mt of *stockwork* ore. The great majority of VMS deposits in the IPB are pyrite-rich, although an important number showed locally high ore grades of base metals, including Cu, Zn and Pb (Leistel et al. 1998, Velasco et al. 1998, Tornos 2006).

The present study considers the lakes that have formed in two abandoned mine pits in the IPB: Cueva de la Mora and Herrerías-Guadiana (Fig. 1.1 to Fig. 1.4). The first one

is located in the northernmost sector of the IPB (Sánchez-España 2000, Tornos 2008), at the west of a village which goes by the same name. The latter is located in the southernmost sector, next to the mine village of Las Herrerías and nearby the Tharsis mine district.

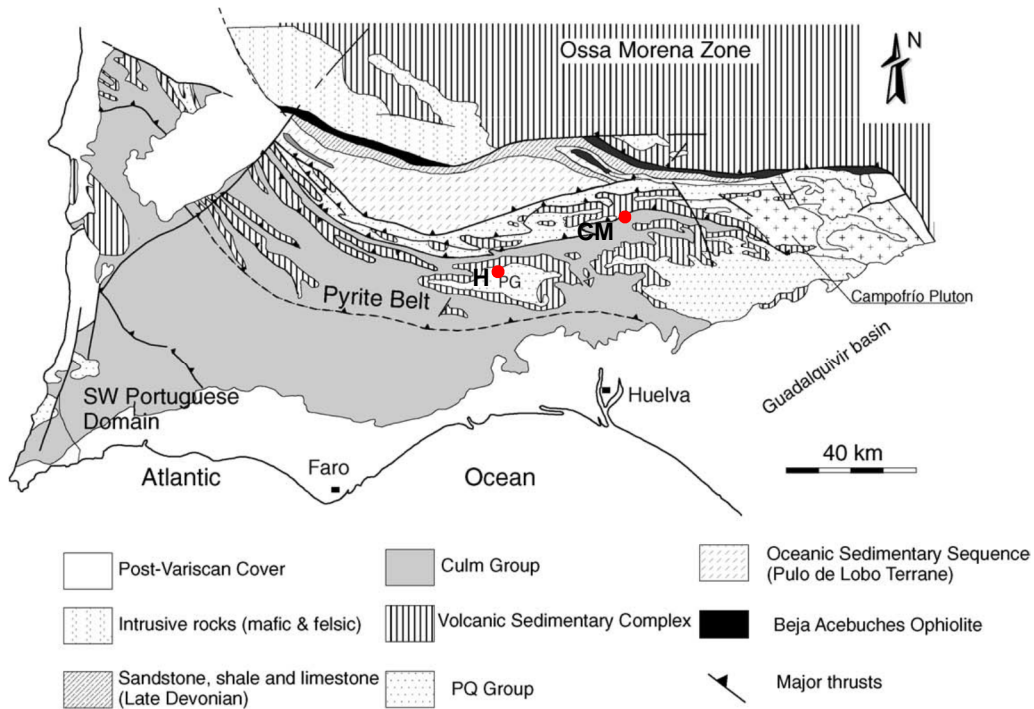


Figure 1.1. Geological framework of the Iberian Pyrite Belt (top), (modified from Tornos 2006). Location of Cueva de la Mora (CM) and Herrerías (H) mines (red dots) and other mines of the IPB (blue dots); SD, São Domingos; ST, San Telmo; Th, Tharsis (bottom; aerial photograph taken from Google Earth).

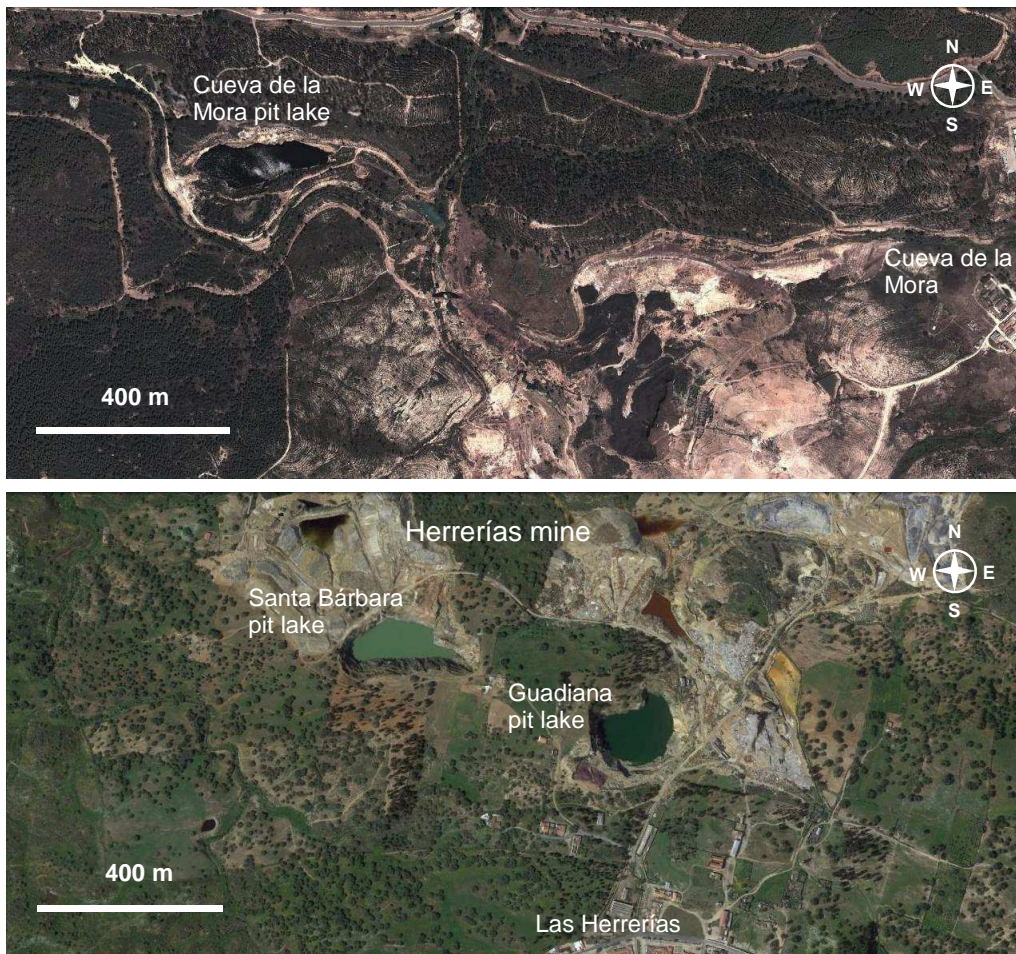


Figure 1.2. Aerial photographs of Cueva de la Mora (top) and Herrerías mines with the two pit lakes (bottom). Photographs taken from Google Earth.

The ore deposit of Cueva de la Mora comprises acid and basic volcanic rocks, with interlayered black shales rich in organic matter. The mineralization consisted in two parallel lentic structures situated at 180 m depth, and the dimensions of the pyritic ore body were: 350 m in length, 60 m in width, with an average thickness of 50 m. The mineral assemblage was mainly dominated by pyrite (Fe_2S), with abundant chalcopyrite (CuFeS_2) and, in a minor extent, sphalerite (ZnS), galena (PbS), arsenopyrite (FeAsS), pyrrhotite (Fe_{1-x}S) and trace magnetite (Fe_3O_4) (Velasco et al. 1998, Sánchez-España 2000). The total reserves (extracted mineral plus ore reserves) were estimated to be 4.2 Mt, with average ore grades of 1.5% Cu, 0.3% Pb and 0.7% Zn (Pinedo Vara 1963).



Figure 1.3. Postcard of the Cueva de la Mora pit when the mine was active (top; picture from <http://lafactoria-cuencaminera.blogspot.co.uk>); photography of the Cueva de la Mora flooded pit in 2011 (bottom).

Las Herrerías mine was divided in two different sectors: Santa Bárbara and Guadiana. The first evidence of mining works (Roman times) was found in the Santa Bárbara sector, although its modern exploitation started in the 19th century. This deposit consisted in Cu-rich secondary minerals (including carbonates, sulphides and native Cu) which were probably formed by the oxidative alteration of a former massive sulphide deposit (Pérez Macías 2008). The Guadiana sector was a massive sulphide deposit and consisted in five ore bodies (the pit ore, and four of 5 m, 8-10 m, 25 m and 5 m thickness). The pyritic ore from this mine was of high quality, and the mineral reserves were estimated to be 3 Mt, with 0.9% Cu, 0.3% Pb and 0.4% Zn (Pinedo Vara 1963, IGME 1982).



Figure 1.4. Postcard of the Guadiana pit when the mine was under exploitation in 1908 (top; picture from <http://lafactoria-cuencaminera.blogspot.co.uk>); photograph of the Guadiana pit lake in 2012 (bottom).

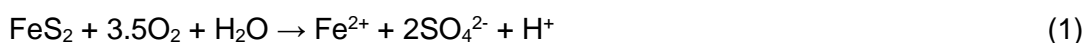
1.2 BASIC CONCEPTS OF ACID MINE DRAINAGE CHEMISTRY

Pyrite is an iron sulphide (FeS_2) that serves as a substrate for many different bacterial species that catalyse the oxidation of sulphur and ferrous iron (Nordstrom and Alpers 1999, Baker and Banfield 2003, Johnson and Hallberg 2003, Johnson and Hallberg 2009, Hallberg 2010). This mineral is the most abundant sulphide in the lithosphere and is usually associated with other sulphides containing industrially important metals (Cu, Zn, Pb), (Johnson and Aguilera 2015). When pyrite is exposed to the oxidizing atmosphere, it is rapidly oxidized by both abiotic and microbial pathways which represent the starting point in the formation of acid mine drainage (AMD),

(Nordstrom 1982, Evangelou 1995, Nordstrom and Southam 1997, Nordstrom and Alpers 1999, Stumm and Morgan 1996, Baker and Banfield 2003, Johnson 2003, Johnson and Hallberg 2003). Pyrite is the main contributor to the formation of AMD in the Iberian Pyrite Belt (IPB), being especially reactive due to its fine-grained nature (Sánchez-España et al. 2005).

When pyrite is exposed to both oxygen and water, spontaneous oxidation begins promoting the formation of acidic metal-rich water. The overall simplified reactions to explain the oxidation of pyrite have been described elsewhere (e.g. Nordstrom 1982, Johnson 2003, and Johnson 2012).

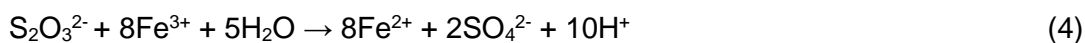
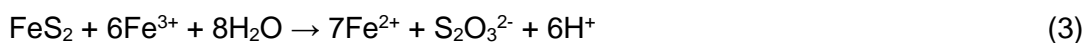
In the first stages, the pyrite exposed to air is chemically attacked by oxygen:



In a second phase, ferrous iron is oxidised to ferric iron:



Rapidly ferric iron attacks the pyrite acting as an oxidant and releasing protons in a two steps reaction:

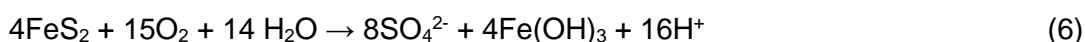


The acidity of the water increases with ferric iron hydrolysis:



At low pH (<4.0) and under abiotic conditions reaction 2 is much slower than reaction 1 suggesting that reaction 2 is the limiting-step for pyrite oxidation (Singer and Stumm 1970). However, the presence of acidophilic microorganisms accelerates the process (reaction 2) generating ferric iron that acts as the chemical oxidant in reaction 3 (and 4), (Nordstrom 1982, Singer and Stumm 1970). Thus, the oxidation of pyrite by ferric iron (reaction 3), is the dominant path to acid mine drainage generation (Johnson and Hallberg 2003, Sánchez-España 2008). The process involves a continuous generation of protons, so that bacterially catalysed sulphide dissolution can drive pH to very low values (Baker and Banfield 2003).

The vast majority of massive sulphide deposits mined in the IPB have generated acidic waters with pH <4.0 (Sánchez-España et al. 2005). However, there are a few exceptions where the presence of carbonates associated with the sulphides and host rocks has buffered and counteracted the acidity released by pyrite dissolution. Examples of the latter case are the pit lakes of Santa Bárbara (Herrerías mine, pH 4.7) and Los Frailes (Aznalcollar, pH 7.2) (Sánchez-España et al. 2008). The balance between acid-generating and acid-consuming reactions determines the pH and acidity load of acidic mine waters as shown in the overall reaction 6 (Blowes et al. 2014).



Acid mine drainage is usually rich in metal(oid)s (e.g. Fe, Al, Cu, Zn, As, Ni, Mn, Cr, Pb). The presence of these metals is determined by the mineralogy of the mined ores and the general tendency is an increase in metal concentration and a decrease in pH (Plumlee et al. 1999). These metals remain in solution because of their high solubility in acidic liquors (Johnson and Hallberg 2003), and their ionic speciation in the acidic waters is usually characterized by their presence as free metal ions or by their aqueous complexation as sulphate complexes (Plumlee et al. 1999).

Some authors have grouped mine drainage waters on the basis of the geological characteristics of the AMD sources (ore type) (Plumlee et al. 1999), though acid mine drainage composition simply depends on the mineralogy and grain size of the mined ores and their host rocks.

1.3 PHYSICO-CHEMICAL CHARACTERISTICS OF ACID PIT LAKES

With respect to natural lakes, pit lakes represent a very special type of lake characterized by high relative depths (depth to surface ratio) which favour meromixis, and by a singular hydrochemistry with low pH and high metal contents (Geller et al. 2013). Pit lakes can also display important differences as regards to nutrient availability, dissolved oxygen content, micro and macro-organisms diversity and abundance, light availability, and many other hydrogeochemical characteristics. The majority of biogeochemical and microbiological research conducted on AMD generation and acidic environments has been focused on streams and rivers affected by acid mine drainage. Good examples are those of the volcanogenic massive sulphide deposits of Cae Coch and Parys Mountain (North Wales), Iron Mountain (California), or the Tinto River (Spain)

(Johnson et al. 1979, Johnson et al. 1993, Hallberg et al. 2006, Kimura et al. 2011, Kay et al. 2013, Nordstrom et al. 2000, Bond et al. 2000, Druschel et al. 2004, López-Archilla and Amils 1999, López-Archilla et al. 2000, González-Toril et al. 2003, 2011, Aguilera et al. 2006, 2007, Souza-Egipsy et al. 2008, de la Torre et al. 2011, Sánchez-Andrea et al. 2011, 2012, García-Moyano et al. 2012). Other examples of extensively studied acid pit lakes are the Berkeley pit lake (Montana, USA), Lake 111 (Lusatia, Germany) and more recently, San Telmo, Cueva de la Mora and Guadiana in the IPB (SW Spain), (Fig. 1.2 to Fig.1.6), (Davis and Ashenberg 1989, Bachmann et al. 2001, Koschorreck and Tittle 2002, Wendt-Potthoff et al. 2002, Karakas et al. 2003, Gammons et al. 2003, 2006, Meier et al. 2004, Gammons and Duaiame 2006, Sánchez-España et al. 2009, 2013, 2014a, 2014b, Diez-Ercilla et al. 2014).



Figure 1.5. Aerial photographs of the Berkeley pit lake (top) and Lake 111 (bottom). Photographs taken from Google earth.

Pit lakes result from the flooding of abandoned open cast mines developed in different mining industries, including coal (lignite), sand (gravel) and metal mining. The different nature of the resource that is mined, as well as many technical (geological, geotechnical) and economic aspects deeply determine the geometry of the pit, and so that the resulting mine pits may largely differ in size, depth and shape. Thus, pits resulting from coal mining usually show large surface extension but relatively shallow depths while metal mining is usually associated with deeper pits with relatively smaller surface areas (Geller et al. 1998, 2013). In the latter case, a significant improvement in metal mining technology in modern times has also resulted in a further increase in the open cast mine's depth and size. The shape of the former void would obviously determine the morphology of the future pit lake. Some lakes are very shallow (e.g. 10 m depth in Mining Lake 111 in the Lusatian mining district, Germany) while other pits can be much deeper (e.g. San Telmo, IPB, 135 m depth). Similarly, pit lakes may range from very large (e.g. Lake Geiseltal, Central German District, 1,853 ha) to very small (e.g. Cueva de la Mora, 1.78 ha), (Bachmann et al. 2001, Sánchez-España et al. 2007a, 2009, Schultze et al. 2013).

The hydrological context of the IPB pit lakes can also differ substantially. Some of the pit lakes have reached hydrological equilibrium and keep a nearly-constant water volume as a result of a balanced water budget with inputs~outputs (e.g. San Telmo, Fig. 1.6), whereas others are still being flooded and show a constant volume increase (e.g. Guadiana in Herrerías mine). In hydrologically balanced lakes, the inputs (groundwater, rainfall, and runoff) are counterbalanced by the outputs (evaporation, water outflows). Surface water inputs can consist in seasonal or ephemeral seepage or a continuous inflow. Pit lakes that have reached hydrological equilibrium are usually older (e.g., Confesionarios, abandoned more than a century ago) or located in areas of hydrogeological recharge (cross-flow lakes, e.g. San Telmo, abandoned in 1989; Fig. 1.6) (Sanchez-España et al. 2008, 2014a). The mines that are still being flooded correspond to the ones where mining activity lasted longer, including some of the biggest mines (e.g. Corta Atalaya, Fig. 1.7; or Aznalcóllar; López-Pamo et al. 2009, Sanchez-España et al. 2008) and some others of small size (Guadiana pit in Herrerías mine; Sánchez-España et al. 2014b). Many pit lakes in the region donate acidic water to the local hydrological basin through old mine galleries (e.g. Concepción, Angostura), waste piles (e.g. Corta Atalaya, Fig. 1.7; or Filón Centro) or as direct discharge (e.g. San Telmo, Fig. 1.6). Therefore, the acidic pit lakes represent important sources of acidic mine water and metal pollution in the surface waters and sediments of the area.



Figure 1.6. San Telmo pit lake: aerial photograph (taken from Google Earth, top) and partial aspect of the pit lake surrounded by waste piles (picture taken in 2011, bottom).

Lakes are generally classified according to their stratification pattern. Depending on the mixing regime, there are two different stratification patterns in which lakes are traditionally grouped: holomictic and meromictic (Wetzel 2001). Holomictic lakes are those in which the water column is completely mixed at least once a year. During the summer, the water column is divided into two layers: an *epilimnion* (the upper layer, warmer due to continuous solar irradiation) is situated above a *hypolimnion* (the bottom layer, cooler and denser). During the winter months, the temperature of the upper layer drops and both upper and bottom layer mix due to density homogenization (Boehrer and Schultze 2009). Aznalcóllar and Santa Bárbara (Herrerías mine) have been both reported to be holomictic (Sánchez-España et al. 2008a). More recently, Nuestra Señora del Carmen has been also reported to present periods of total homogenization during the coldest months (Santofimia et al. 2012)



Figure 1.7. Cerro Colorado (top) and Corta Atalaya pit lakes (picture taken in 2006; bottom).

On the other hand, meromictic lakes are those in which the water column is perennially divided into two layers that never mix. The oxic upper layer is the *mixolimnion* and the anoxic bottom layer is the *monimolimnion*. The mixolimnion is in contact with the oxidising atmosphere, and hence it is oxygen-rich, and it is under seasonally variable weather variations. The monimolimnion is depleted in oxygen, and completely isolated from the atmosphere.

At the same time, meromictic pit lakes of the IPB have been divided in Type I and Type II. The Type I pit lakes (e.g. San Telmo) are characterized by a homogeneous monimolimnion with virtually no vertical change in water chemistry, whilst a sub-stratified bottom layer characterizes Type II pit lakes (e.g. Cueva de la Mora and Guadiana)

(Sánchez-España et al. 2008a, 2009, 2013). The monimolimnion in these Type II pit lakes show marked vertical gradients of pH and metal concentration as a response to a complex flooding history and to increasing sulphide/water interaction at depth.

The majority of pit lakes in the IPB show low pH in the upper part of the water column, from extremely acidic (e.g. Corta Atalaya, pH 1.2 in 2006) to highly acidic (pH 2.2 to 3.6, e.g. San Telmo, pH 2.6-2.8 in 2011). There are a few exceptions with pH higher than 4.0, such as Santa Bárbara in Herrerías mine (pH 4.7; Fig. 1.8) and Los Frailes (pH 7.2) (Sánchez-España et al. 2008a, 2013). In these two lakes, the relatively high pH values are caused by the scarcity of pyrite in the host rock and by the presence of carbonates and/or alkalinity counteracting the acidity released by sulphide oxidation.



Figure 1.8. Santa Barbara pit lake (Herrerías mine).

The presence of dissolved metals and sulphate is determined by the water acidity (Fig. 1.9). Dissolved metals in mine pit lakes are usually present in high concentration (Table 1.1). Besides pH, the high amount of iron is also determined by the lithology of the rock substrate, e.g. mainly the pyrite content. Other metals and metalloids may be also present due to the dissolution of other sulphide minerals like sphalerite (Zn), chalcopyrite (Cu, Fe), galena (Pb), and arsenopyrite (As, Fe), aluminosilicates like

chlorite and muscovite (Al, Fe, Mg, Co, Ni) or feldspars (Al, K, Na, Ca), and other minerals (Sánchez-España et al. 2008a).

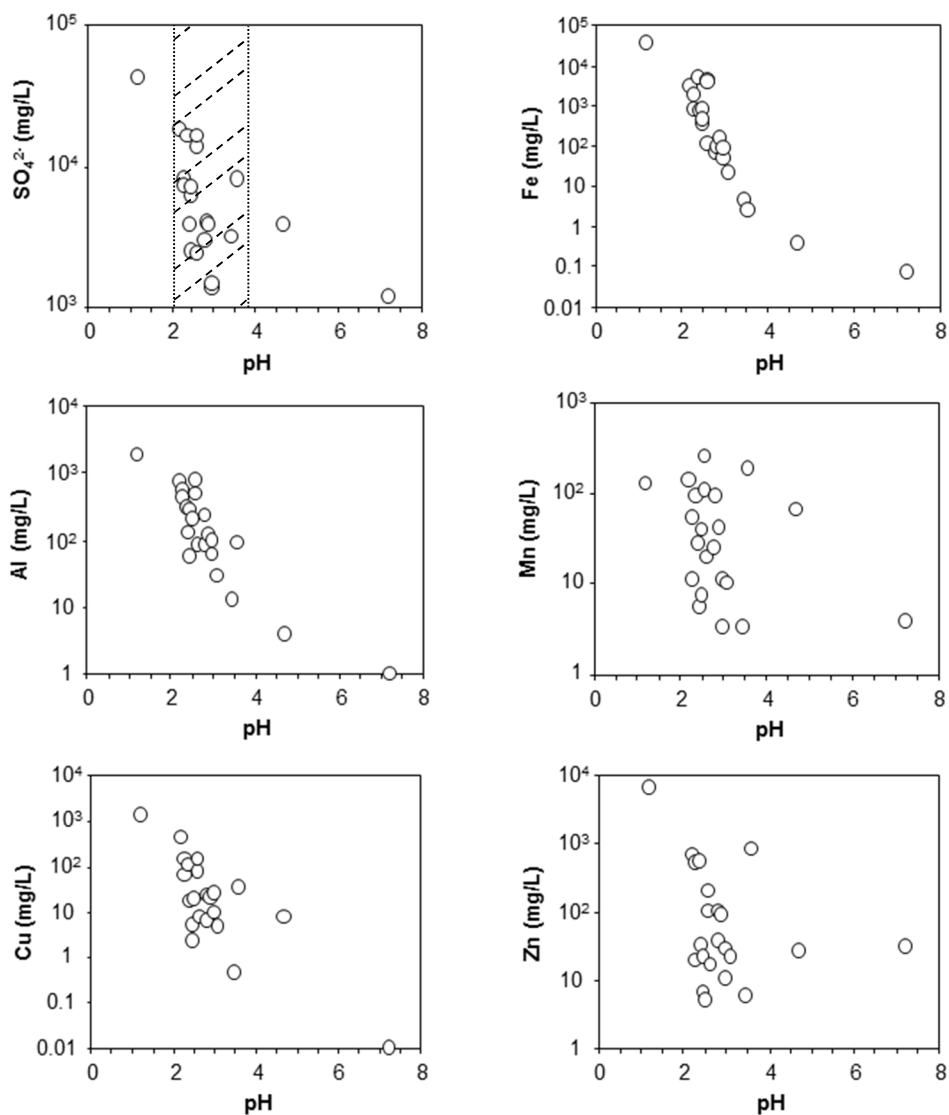


Figure 1.9. pH-dependent variation of sulphate and dissolved metals in surface water of the Spanish pit lakes of the IPB (modified from Sánchez-España et al. 2013).

The precipitation of schwertmannite and felsöbányaite involves the release of protons whilst their dissolution requires the consumption of protons. In acid mining lakes, the formation and dissolution of these two minerals act as pH-buffering systems at the pH values at which they are formed (pH 2.5-3.5 for schwertmannite and 4.0-5.0 for felsöbányaite). The precipitation of both minerals usually involves co-precipitation of other metals (Uhlmann et al. 2004, Sánchez-España et al. 2005, 2011).

Table 1.1. Metal concentration in different pit lakes in the world and the Tinto River as a natural acidic environment. *Sánchez-España, personal communication. References (Ref.): (1) Sánchez-España et al. 2007a, (2) González-Toril et al. 2009, (3) López-Archilla et al. 2001, (4) Koschorreck 2013, (5) Kumar et al. 2013, (6) Gammons and Duaiame 2006, (7) Moser and Weisse 2011. All the concentrations are in mg/L.

Lake	Country	Stratification type	Mineral extracted	Max depth (m)		pH	E _H (mV)
San Telmo	Spain	Meromictic type I	Pyrite	135	Mixolimnion	2.9-2.6*	777-877*
					Monimolimnion	2.9*	640*
Tinto river	Spain					2.0-2.5	464-530
Lake 111	Germany	Seasonal stratification	Lignite	10.5		2.6	400-650
Mount Morgan	Australia	n.d.	Gold, copper	n.d.		2.8	n.d.
Berkeley pit lake	USA	Meromictic	Copper	210	Mixolimnion	2.75	648
					Monimolimnion	2.5	604
Acid mine lake at Langau	Austria	Meromictic	Coal	9.7	Mixolimnion	2.6	n.d.
					Monimolimnion	3.6	n.d.

Table 1.1. Continued.

Lake		Mg	Fe	Al	Cu	Zn	As	Ni	Ref.
San Telmo	Mixolimnion	484	155	140	24	89	0.1	0.5	1
	Monimolimnion	615	171	150	30	116	0.1	0.6	
Tinto river		n.d.	2450	81	22-119	225	n.d.	9.3	2, 3
Lake 111		30	171	38	0.006	1	n.d.	0.2	4
Mount Morgan		1240	248	740	36	25	n.d.	n.d.	5
Berkeley pit lake	Mixolimnion	534	564	275	79	627	0.1	2.4	6
	Monimolimnion	515	1007	268	153	638	0.1	1.6	
Acid mine lake at Langau	Mixolimnion	64	81	14	0.04	0.5	n.d.	n.d.	7
	Monimolimnion	133	133	5	<0.02	0.2	n.d.	n.d.	

1.4 MICROBIOLOGY IN ACIDIC ENVIRONMENTS

The diversity of microorganisms in nature is extremely large, although they can be classified phylogenetically (by DNA similarity). The scientific community has tried to classify them into different categories according to their physiological characteristics (e.g. tolerance to temperature or pH, oxygen availability, radiation intensity, and tolerance to dissolved salt concentration) or their metabolic requirements (carbon and energy source), (Prescott et al. 1999, Madigan et al. 2014).

Depending on the carbon source (organic or inorganic), microorganisms can be classified as autotrophs (inorganic carbon, CO₂) or heterotrophs (organic carbon), and also classified on the basis of their energy source (Table 1.2). Some microorganisms that are able to use an inorganic compound as an electron donor and need an organic carbon source, these are facultative heterotrophs (Madigan et al. 2014). For example the algae *Chlamydomonas acidophila* or *Ochromonas* sp. that do photosynthesis but are able to use organic resources and some species of the sulphur-oxidizing bacteria genera *Beggiatoa* spp. (Tittel et al. 2009, Andersson et al. 1989, Jones et al. 2000, Madigan et al. 2014).

Table 1.2. Bacterial nutritional groups and the type of energy and hydrogen/electron source. Modified from Prescott et al. (2009).

Nutritional group	Energy source	Hydrogen/electron source
Phototroph	Light	Inorganic
Chemolithotroph	Inorganic chemicals	Inorganic
Chemoorganotroph	Organic chemicals	Organic

Microorganisms play an important role in rock and mineral weathering by the alteration of the reactive mineral constituents, by oxidation or reduction, or by the formation of metabolic products (Fig. 1.10). Microbes even play an important role in the formation of minerals or sedimentary rocks as various types of microbialites in carbonate environments (Ehrlich 1998, Konhauser 1998, Riding 2011). Microorganisms inhabiting extreme environments have been deeply studied for several decades; many reviews have been published about extremophiles (e.g. Horikoshi and Grant 1998, Gerday, and

Glansdorff 2007) providing overviews of the different groups of extremophiles. Those inhabiting acidic environments are referred to as *acidophiles*, and have been under intense research for decades (Norris and Ingledew 1992, Norris and Johnson 1998, Johnson 2007, 2009, 2011). The most extensively studied acidophile is *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans*) which was discovered in the 1940s and described in the early 1950s (Temple and Colmer 1951).



Figure 1.10. Pyrite rich rock showing pyrite crystals (left) and the microbially attacked surface (right) collected from the adjacent area to the Tinto river source.

Acidophilic microorganisms are widespread among the three Domains of life (Johnson 2006): *Eukarya*, *Bacteria* and *Archaea*. These microorganisms need to confront the low pH conditions and the high metal concentrations typical of these extreme environments. Moreover, the temperature in these extreme environments can sometimes be extreme ranging from $<0^{\circ}\text{C}$ to $>80^{\circ}\text{C}$ (Johnson 2012). These extremophilic microorganisms, mainly bacteria, can be classified according to their pH-tolerance: extreme acidophiles (optimal growth at $\text{pH} < 3$); moderate acidophiles (optimal growth at $\text{pH} 3\text{-}5$); and acid-tolerant bacteria (optimal growth at $\text{pH} > 5$) (Johnson 1998, 2012). Acidophilic prokaryotes can also be classified in terms of their optimum growth temperature as mesophilic acidophiles (between 20 and 40°C ; mainly Gram-negative bacteria), moderate thermophiles (between 40 and 60°C ; mainly Gram-positive acidophiles), and extreme thermophiles (above 60°C ; exclusively archaea). Although no psychrophilic acidophiles (optimum growth at temperatures lower than 5°C) have been described, there are some exceptions known to be psychro-tolerant, *Acidithiobacillus*

ferrivorans and “*Ferroplasma myxofaciens*” which are metabolically active at 5°C (Hallberg et al. 2010, Johnson et al. 2014).

The dominant metabolism in acidic environments and the most studied one is chemolitho-autotrophy (*Bacteria* and *Archaea*). The abundance of this metabolism is explained by the large quantity of the inorganic energy sources available for microorganisms inhabiting these waters rather than organic carbon. Chemolitho-autotrophy bacteria and archaea use an inorganic energy source, an inorganic electron donor (ferrous iron, sulphur or hydrogen) and CO₂ as carbon source. On the other hand, obligate or facultative heterotrophs need an organic energy source (coming from the lysis of other microorganisms, eukaryotic primary production, or dissolved organic matter), and an organic electron donor and an organic carbon source that is less abundant in these extreme environments. Fermentative metabolisms are rare in these environments probably due to the fact that fermentation products are toxic at low pH. The presence of other metabolisms, like methanogenesis or ammonia-oxidation, is not as clear as the presence of other metabolisms previously mentioned. However, acidophilic methanogens and ammonia-oxidizers are believed to inhabit acidic environment as evidence of both metabolic groups was found in sediments of an acidic mine pit lake (Wendt-Potthoff et al. 2012, Falagán et al. 2014). In addition, an isolate of the archaeal group *Methanobacteriaceae* was obtained from an acidic peat bog sample (Kotsyurbenko et al. 2007) and the ammonia-oxidizing archaea, *Nitrosotalea devanattera*, was isolated from acidic agricultural soil (Lehtovirta-Morley et al. 2011).

The most widespread terminal electron acceptor is oxygen (O₂ (g)) although other electron acceptors have been found to be important under oxygen-deficient conditions, namely ferric iron, sulphate (very abundant in acidic waters, used by sulphate reducers), and sulphur.

Iron oxidation (chemolitho-autotrophy) is a process where ferrous iron (electron donor) is oxidized to ferric iron in the presence of oxygen. Iron oxidizers can obtain carbon by fixing CO₂, (e.g. *Acidithiobacillus* (*At.*) *ferrooxidans*), from organic carbon (e.g. *Ferrithrix thermotolerans*, *Ferrimicrobium acidophilum*), (Johnson et al. 2009) or from both (e.g. *Acidimicrobium ferrooxidans*; Clark and Norris 1996).

Iron reduction is widespread within acidophiles (Johnson and Bridge 2002). Many heterotrophs are able to reduce ferric iron to ferrous iron under microaerobic (*Acidicapsa*

sp. strain MCF9, Chapter 4) or anaerobic conditions (*Acidocella aromatica*, Jones et al. 2013; *Acidibacter ferrireducens*, Falagán and Johnson 2014).

Some acidophilic bacteria are able to grow via the dissimilatory oxidation of hydrogen under aerobic or/and anaerobic conditions, and can couple ferric iron reduction to hydrogen oxidation with a similar growth rate to that in aerobic conditions with hydrogen (Hedrich and Johnson 2013a, 2013b).

Many acidophiles are able to oxidize sulphur to sulphate or polythionates. Sulphur oxidation is very common within the members of the genus *Acidithiobacillus*. However, some acidophilic archaea and *At. ferrooxidans* can perform sulphur disproportionation. This means that sulphur (S^0) is simultaneously oxidized and reduced generating both an oxidized and a reduced form of sulphur (SO_4^{2-} and H_2S), (Osorio et al. 2013).

Whilst just four genera of acidophilic archaea able to reduce elemental sulphur have been described (Johnson and Hallberg 2009), sulphate-reduction is more extended within acidophilic environments. Sulphate-reducing acidophiles have been also identified, although there are more acid-tolerant sulphate reducing bacteria (SRB) than acidophilic SRB, as the optimum pH for growth is usually greater than 5.0 (e.g. *Desulfosporosinus acidiphilus*, Alazard et al. 2010; *Desulfosporosinus acididurans*, Sánchez-Andrea et al. 2014), (Dopson and Johnson 2012). However, scientists have argued that the low availability of organic carbon at low pH limits more significantly the growth of sulphate-reducing bacteria rather than the high acidity (Koschorreck 2008). Sulphate is used to oxidize the organic carbon source (e.g. glycerol), which can be reduced completely to CO_2 or partially to acetic acid that is fairly toxic under low pH conditions (pK_a 4.75, at pH <4.75, acetic acid is present in its acid form whereas at pH >4.75 is present as acetate). This could explain why less acidophilic SRB have been described.

There are extreme (*Acidiphilium* sp.) and moderate (*Acidisphaera rubrifaciens*) acidophiles within the heterotrophs (use organic carbon for growth). The carbon source is usually in the form of dissolved organic carbon provided from the primary producers (phototrophs and chemolithotrophs) inhabiting these acidic environments. The range of organic carbon compounds used by acidophilic heterotrophs is quite narrow and includes monomeric sugars, alcohols and amino acids. Aliphatic acids are in general toxic to acidophiles, although e.g. *Acidocella aromatica* is able to grow on acetic acid (Jones et al. 2013).

Within bacteria phyla, acidophiles are spread into the *Proteobacteria* (α -, β -, and γ -*Proteobacteria*), *Nitrospira*, *Aquificae*, *Verrucomicrobia*, and *Actinobacteriaceae*. Within *Archaea*, acidophiles include species belonging to the *Euryarcheota* and *Crenarcheota* (Johnson and Aguilera 2015).

Eukaryotes in acidic environments are distributed among microalgae, protozoa, yeast, fungi, and rotifera. The eukaryotic community in acidic environments is mainly distributed in biofilms in streams and rivers. There are phototrophs such as filamentous algae, diatoms, photosynthetic protists, microalgae and protozoa in addition to facultative heterotrophs. Higher in the food chain it is possible to find ciliates, flagellated, amoeba and heliozoans which are less abundant. They are usually acid-tolerant rather than acidophilic organisms. Normally, in acid pit lakes, flagellated facultative heterotrophs are abundant because the scarceness of dissolved CO₂ makes necessary to search for this resource through the water column. Generally, the higher the pH, the more phototrophs are present in these acid ecosystems; the presence of algae in acid environments is determined by the availability of phosphorus, light, and presence of metals. Phytoplankton develops strategies of adaptation to low pH environments, high proton and metal concentration, and low nutrient concentration (Johnson and Aguilera 2015).

Fungi and yeasts have been less widely studied than other microorganisms inhabiting acid environments. Nevertheless, they are highly acid tolerant, and some fungal species are able to even grow at pH 0, although they can also grow at neutral and alkaline pH (Johnson and Aguilera 2015).

1.5 MICROBIOLOGY OF ACIDIC PIT LAKES

Bacterial composition of acidic pit lakes is highly variable. Bacterial cell numbers in German acidic pit lakes are rather constant in time and positively correlated with dissolved organic carbon content, while negatively correlated with pH (Wendt-Potthoff 2013). Taxonomic composition of bacterioplankton in acidic pit lakes is scarce. The communities are formed by α -, β - and γ -*Proteobacteria*, as well as *Actinobacteria* and *Acidobacteria*. Lake 111 in the Lusatia mining district has been largely studied, its water column is fully oxygenated, and therefore no anaerobic microorganisms have been detected. The dominant bacterial groups in this lake are *Nitrosomonas* (β -*Proteobacteria*), *Acidobacteria*, *Acidiphilium* (α -*Proteobacteria*), ammonia oxidizers, and *Legionellae* (γ -*Proteobacteria*), (Wendt-Potthoff 2013). Little is known about the bacterial

composition of other acidic pit lakes present in Australia or in the Berkeley pit lake in USA. In the most Austrian acidic lake (lake in Langau), filamentous bacteria were the most abundant morphological bacterial type within the bacterial community (Wendt-Potthoff 2013).

Acidic pit lakes of the Lusatian mining district (Germany) showing a pH <3.5 are generally colonized by microorganisms belonging to the *Chrysophyceae*, *Chlorophyta*, *Bacillariophyceae*, *Euglenophyceae*, and *Dinophyceae* (Nixdorf et al. 2001) and less commonly, *Trebouxiophyceae* and *Prasinophyceae*. Phytoplankton communities in these lakes changes when the pH increases (Wendt-Potthoff 2013). Facultative heterotrophy is common in phototrophs that inhabit acidic mine pit lakes, *Chlamydomonas acidophila* and *Ochromonas* sp. are the main facultative heterotrophs present in these environments (Moser and Weisse 2011). Although these are generalizations, phytoplankton composition is very variable seasonally and spatially, e.g. *Lepocinclis* was responsible of a high chlorophyll maximum in the Austrian lakes whereas *Chlamydomonas* sp. caused other chlorophyll event at Lake Waldsee in Germany (Moser and Weisse 2011).

Zooplankton diversity is even lower than that of phytoplankton in acidic pit lakes. Very few species can tolerate the very extreme conditions in these acidic environments. Wendt-Potthoff (2013) provides an extensive review of the zooplankton species found in acid pit lakes in Germany and USA. The main groups found within these lakes are *Rotifera* (*Monogononta*, *Bdelloidea*), *Cladocera* (*Chydoridae*, *Daphniidae*, *Bosminidae*), and *Copepoda* (*Cyclopidae*). There is a lack of knowledge about the physiological adaptations of zooplanktonic species to acidic conditions. Zooplankton community in acidic pit lakes is determined by pH and metal concentrations, while biomass is determined by food availability (Wendt-Potthoff 2013). Heliozoan, ciliates, amoebae and nanoflagellates are also found in acidic pit lakes. For example, in the Austrian acidic mining lake, the heliozoan *Actinophrys sol* is barely present due to the food scarcity of phytoplankton, on which it feeds (Moser and Weisse 2011). Within ciliates, *Prostomatida*, *Hypotrichida* and *Peritrichida* have been detected together with the nanoflagellated cercomonad. In general, protozooplankton diversity is low and the main component of the protozoan community varies during the year (Wendt-Potthoff 2013).

Fungi and yeast occurrence in acidic pit lakes has not been studied as thoroughly as in other similar environments (acidic streamers or rivers), but they are widely present

in acid lakes and usually appear in enrichments or plates when cultivating water samples in the presence of organic carbon. Gross and Robbins (2000) provide a list of more than 80 fungal species found in acid mine drainage environments.

Microbial population in sediments is usually slightly different from that in the water column and, moreover, bacterial numbers are usually greater in sediments than in the water column. In addition, sediments at intermediate depth show more diversity than those at shallow or deep depths, which is commonly ascribed to the low organic carbon availability at depth. Although similar bacterial groups are found in lake waters and sediments, some species typically present in pit lake sediments have not been found in the water column.

1.6 MICROBIOLOGY IN THE IPB

The microbiology present in the acid environments of the IPB lakes has been poorly studied until recent years. In contrast, the Tinto River is one of the most studied acidic environments in the world (López-Archilla and Amils 1999, López-Archilla et al. 2000, Aguilera et al. 2006, García-Moyano et al. 2007, 2012, Amils et al. 2007, 2011, González-Toril et al. 2003, 2009, 2010, 2011, Gómez-Ortiz et al. 2014). However, there are not many studies focused on the microbiology of other acid environments in the IPB, and the existing ones are not very thorough (e.g. Bryan et al. 2006, Rowe et al. 2007, Sánchez-España et al. 2007a, 2007b, 2008, González-Toril et al. 2011).

1.6.1 MICROBIOLOGY OF THE TINTO RIVER

The Tinto River (Fig. 1.11) has been proposed as an analogous of Mars environment (Amils et al. 2011) and considered as a natural acid environment (Gómez-Ortiz et al. 2014). The main contributors to the microbial biomass of the water column in the Tinto basin belong to three bacterial genera, *Leptospirillum*, *Acidithiobacillus* and *Acidiphilium*, although bacterial community changes along the course of the river (González-Toril et al. 2003). Microbial biomass in this river is also concentrated in macrofilaments floating on the water surface where bacterial and archaea coexist enmeshed in an organic matter-rich matrix with mineral particles (García-Moyano et al. 2007). The most important metabolism in the river water is iron oxidation, but iron reduction and oxidation of reduced sulphur compounds are also very important

metabolisms contributing to the chemical processes in the river water (González-Toril et al. 2003).



Figure 1.11. The Tinto River.

Sediments of the Tinto river are more microbially diverse and show a higher bacterial biomass compared with the river waters (García-Moyano et al. 2012). The microbial ecosystem is dominated by bacteria although archaeal species are also present (Sánchez et al. 2011) but no evidences of eukaryotic cells can be found (Sánchez-Andrea et al. 2012). The main characteristic bacterial groups found in AMD are also present in sediments of the Tinto River, as well as other that are not so common as *Cyanobacteria*. In the sediments of the Tinto river, iron oxidation, iron reduction, oxidation of reduced sulphur compounds and heterotrophy have been found in addition to sulphate-reducers (Sánchez-Andrea et al. 2011, 2013) and methanogens in deep sediments (>20 cm deep from the sediment-water interface; Sánchez et al. 2011). A microbial ecological model has been proposed in which the most acidophilic metabolisms (iron oxidation, iron reduction and heterotrophy) are dominant in shallow sediments, whilst sulphate-reduction, methanogenesis, nitrate-reduction and fermentative metabolisms are mainly present in deeper sediments with higher pH (Sánchez-Andrea et al. 2011).

The eukaryotic community in the Tinto River, mainly formed by *Dunaliella*, *Euglena* and *Chlamydomonas*, is influenced by pH and the presence of dissolved metals, being mainly concentrated in biofilms along the riverbed (Aguilera et al. 2006) which are also colonized by bacteria and archaea (Souza-Egipsy et al. 2008). Other eukaryotes such as *Cyanidium*, *Chlorella*, filamentous algae (*Zygnemopsis*, *Klepsormidium*), the diatom *Pinnularia* and heliozoan, amoeba, ciliates, flagellates and rotifer as well as fungi are also present in these biofilms (Aguilera et al. 2007).

1.6.2 MICROBIOLOGY OF IBERIAN PIT LAKES

The microbiology of some acidic mining lakes (San Telmo, Cueva de la Mora, Nuestra Señora del Carmen, and Concepción) in the IPB has been recently described in Sánchez-España 2007a, 2008, Wendt-Potthoff et al. (2012) and in Santofimia et al. (2013). These lakes differ greatly in terms of physico-chemical features, the lake column is divided into different layers, the two principal layers being an oxic upper layer and an anoxic bottom layer. The microbial population inhabiting these layers is determined mainly by the presence of light and oxygen. The bacterial and archaeal diversity in the San Telmo pit lake has only received superficial attention, and just the main bacterial groups were identified (α - and γ -*Proteobacteria* and *Leptospirillum*). The upper layer (mixolimnion) in this pit lake is the most densely populated (higher biomass) although presents very low diversity, whilst the opposite occurs in the middle layer (chemocline). In the anoxic bottom layer (monimolimnion) the biomass and the biodiversity increases with respect to the chemocline. Archaea are found in the chemocline and in the mixolimnion although in low numbers if compared with bacterial biomass (Sánchez-España et al. 2007a). Near the lake, a very extreme acidic (pH 0.6-0.8) leachate seeps from a pyrite pile and ends in a green pool in the San Telmo mine. The microbial community is dominated by *Ferroplasma* sp. (51.5% of the total microbial biomass) whilst *Thermoplasma* sp., and α -*Proteobacteria* and the bacterial genera *Acidithiobacillus* sp., *Leptospirillum* sp. represent the 49.5% (Sánchez-España et al. 2008). The Cueva de la Mora pit lake upper layer is populated by phytoplankton and bacteria (iron oxidizers, iron reducers and sulphur oxidizers), phytoplankton disappears in the transition layer (separating the upper oxic and the lower anoxic layers) and in the lower layer whilst iron reduction and sulphate-reduction metabolism are detected. Microbial metabolisms distribution in Nuestra Señora del Carmen is similar to that found in Cueva de la Mora, although no sulphate-reduction has been found in the water column. The case of the

Concepción pit lake is a little different as this lake is dominated by heterotrophs, iron oxidizers, iron reducers, and, just in the deepest part of the lake, sulphur oxidizers. The lake water mass undergoes periods of mixing making, so that it is possible to find oxygen dependant metabolisms in the whole water column. The sediments present similar bacterial metabolisms also present in the lake water. In Nuestra Señora del Carmen and in Concepción, the microbial population composition has been determined by cloning. The typical species populating AMD environments are also present in the water column of these two lakes (*Acidithiobacillus*, *Leptospirillum*, *Acidiphilium*, *Acidisphaera* and acidophilic actinobacteria), in addition to archaea (*Thermoplasmatales*) and the phototroph *Chlamydomonas* (surface water) (Santofimia et al. 2013). Similar bacterial species present in Nuestra Señora del Carmen are found in Concepción pit lake, although *Acidithiobacillus* and *Leptospirillum*, the two most characteristic species of AMD, were absent from the latter.

Although in acid environments the microbial populations are similar, they differ from one place to another, as the microorganisms inhabiting these extreme environments are influenced by physico-chemical parameters. Thus, iron oxidizers, iron reducers, sulphur oxidizers and, in the anoxic sediments, sulphate-reducers are present in the IPB acid lakes, rivers and streams (e.g. Rowe et al. 2007, Sánchez-España et al. 2007a, López-Archilla and Amils 1999).

Notwithstanding the studies mentioned above the microbial community present in acidic pit lakes is still poorly known.

1.7 GENERAL CONCEPTS OF BIOMINING

The use of prokaryotic microorganisms to facilitate the recovery of metals from ores and waste materials is known as “Biomining”.

Biohydrometallurgy has been used in the mining industry for several decades. It is technically and economically suitable, and widely employed to process low-grade and refractory ores because the high grade and easily processed ores are becoming less abundant (Brierley and Brierley 2013). It is also considered an environmentally friendly mechanism that needs less high temperatures and leaves a low carbon footprint (Johnson 2014). In biomining, the microorganisms used are autotrophs, which use CO₂ as carbon source, whereas in smelting operation large amounts of CO₂ are produced.

The working temperatures in bioprocessing of minerals are between 20 and 80°C, therefore, no external heat source is required, and, sometimes, it is even necessary to cool down the systems to temperatures suitable for microorganisms. Other advantages of biomining over other processes include: (i) the presence of arsenic in ores, which is kept in solution while bioprocessing but released as gas emission during smelting, and (ii) recovering of by-products that are more easily processed than those in waste materials coming from pyrometallurgy (Johnson 2014). Biomining can be divided into two different processes: bioleaching and mineral biooxidation. The first one is based on the solubilisation of base metals (Zn, Cu and Ni) while the second involves making precious metals (mainly Ag and Au) accessible to chemical extraction by removing (using acidophilic bacteria) minerals such as pyrite or arsenopyrite with which they are associated (Brierley and Brierley 2013).

Bioleaching and biooxidation are currently applied to two different mineral processing mechanisms: heaps and stirred tanks. Heap bioleaching and mineral oxidation are based on stacking fragmented ores onto lined pads. The ore stacks are irrigated with sulphuric acid solutions thereby promoting the growth of microorganisms present in the ore or the microorganisms intentionally added. In the case of base metal ores the solution coming out the heap is called pregnant leaching solution (PLS). The metal is recovered from the PLS by using conventional metal recovery processes (e.g. solvent extraction and electro-winning; SX-EW). In the case of precious metal-containing ores, when enough sulphide mineral is oxidized, the leached ore is removed from the tank, neutralized and treated with other solutions (e.g. cyanide solution) to solubilize the precious metals. Normally, the processes of stirred-tank bioleaching and mineral biooxidation are applied to mineral concentrates. A concentrate is an ore that has been upgraded by flotation or gravity to separate the ore bearing metal from those that do not contain the valuable metals (Johnson 2010, Brierley and Brierley 2013).

Another approach to extract metals is known as *in situ* biomining, in which a ferric iron-rich acid leach liquor is injected to an ore body, which is being disrupted. The metal containing liquor is then pumped out and the metals recovered. The acid liquor could in theory be free of microorganisms or inoculated with a bacterial consortium to favour the leaching process (Johnson 2010). There has been much research into how existing biomining practices can be improved, as well to develop new methods to bioprocess metal ores (Hedrich and Johnson 2014, Rawlings and Johnson 2007, Gericke et al.

2010, Brierley and Brierley 2013) and bioremediate acid mine waters (Ñancuqueo and Johnson 2012b).

Microorganisms are a very important component of these biotechnological processes. During the last decades, there has been an important surge in studies of microorganisms that can be used for biomining. New research on microorganisms and their metabolisms and possible application to biomining has led to the discovery and description of genera and species used in biomining. The main role of microbial populations in biomining is the regeneration of ferric iron by ferrous oxidation and the production of acidity by sulphur oxidation (Rawlings and Johnson 2007). The main problems with the efficiency of heaps leached by microbial populations are the control of pH, aeration, temperature, dissolved solutes, and the need to irrigate the ore. In stirred-tank operations, the accumulation of toxic components, physical damage of cells and shear stress are particularly problematic (Watling 2011).

The predominant microorganisms involved in biomining are extremely acidophilic bacteria and archaea which are able to grow under pH <3, although they can be thermophilic or mesophilic. Within bacteria, species used for bioleaching are found in different phyla (*Proteobacteria*, *Nitrospirae*, *Firmicutes*, and *Actinobacteria*); while within archaea, the majority belongs to two groups, *Sulfolobales* and *Thermoplasmatales* (Johnson 2014). The study of microorganisms suitable for biomining is receiving increasing attention (Norris 1997, Norris et al. 2000, Okibe and Bouchez et al. 2006, Johnson and Roberto 1997, Foucher et al. 2003, Johnson 2004, 2008, Dopson and Lindström 2004, Rawlings 2005, Heinzl et al. 2009, Fu et al. 2013, Johnson et al. 2013, Norris et al. 2013, Watling et al. 2014, Acosta et al. 2014). Metal resources for the global industries will be probably a major problem of our society because of the high demand for metals by emergent Asian countries and the use of an increasingly diverse number of metals in modern applications. The understanding of the whole biomining process is a major issue at present, and a better understanding of the control factors and the microorganisms involved will be crucial for a more efficient recovery of metals in the near future.

1.8 OBJECTIVES

Within the context of a larger research project focused on the formation and nature of the acidic pit lakes of the IPB (Biotic and Abiotic Controls of Chemical Underwater Stratification of Acidic Pit Lakes of the Iberian Pyrite Belt, Reference number CGL2009-09070, funded by the Spanish Ministry of Science and Innovation through its National Research Program), this PhD thesis aims at improving the existing knowledge on the microbiology of these acidic lakes. In particular, the present study is designed to achieve the following objectives:

- To detect the main bacterial metabolisms controlling the lake chemistry and influencing the redox transformations and biogeochemical cycling of key elements as iron, sulphur, carbon, phosphorus and nitrogen.
- To identify the main species of microorganisms present in the lakes Cueva de la Mora and Guadiana, including prokaryotes (bacteria, archaea) and eukaryotes (microalgae composing the phytoplanktonic community and acting as primary producers).
- To characterize the main bacterial species with regard to their morphology, nutritional and energetic needs, metabolic pathways and tolerance to physico-chemical factors such as pH, temperature, and different metal concentrations.
- To enlarge the currently available international databases of bacterial acidophilic and acid-tolerant species inhabiting extremely acidic environments with novel and previously unrecognized bacterial species.
- To improve the present knowledge of microbial ecology in extremely acidic lakes formed in abandoned metal mines, looking at differences and similarities with other acidophilic environments described in the literature.
- To study the interactions of bacterial cells with metal ions in the aqueous solutions, with particular emphasis on the possible influence of bacteria on the mobility of aluminium and their capacity to produce Al biomineralization.
- To evaluate the potential applicability of acidophilic bacterial species for biotechnological applications, particularly with regard to their possible use in copper biomining.

**PHYSICO-CHEMICAL
CHARACTERIZATION OF
CUEVA DE LA MORA AND
GUADIANA PIT LAKES**

2

2.1 INTRODUCTION.....	36
2.2 MATERIAL AND METHODS	37
2.2.1 STUDY SITES AND SAMPLING TECHNIQUES	37
2.2.2 LABORATORY-BASED CHEMICAL ANALYSES.....	40
2.3 RESULTS.....	40
2.3.1 WATER COLUMN CHEMISTRY	41
2.3.2 SEDIMENT CHEMISTRY	46
2.4 DISCUSSION	47
2.5 CONCLUSIONS.....	51

Abstract

Cueva de la Mora and Guadiana pit lakes are meromictic with vertical gradients of water chemistry and redox conditions. The lakes water columns are divided in two main layers, the upper oxic layer, which experiences periods of stratification during the warmer months, and the bottom anoxic layer, which is completely isolated from the atmosphere. The Cueva de la Mora water column presents a very specific stratification pattern caused by underground connections with the mine tunnels. The stratification pattern of the Guadiana water column is influenced by the climatology at the shallower layers and also by groundwater inflow at the bottom of the lake. The transitional layer (which separates the upper and the bottom layers) is very narrow and presents sharp gradients of pH, redox potential, and dissolved oxygen content. Both lakes are characterized by high metal concentrations (e.g. Fe, Al, Mn, Cu, Zn, Co, Ni) in the water column and by relatively low nutrients (P and N) concentrations. Sediments in the Cueva de la Mora pit lake present different physico-chemical features depending on the depth (shore vs. deep basin sediments) and the geochemistry of its pore waters can substantially vary from that of the overlying water column.

2.1 INTRODUCTION

Acidic pit lakes have been extensively studied in the last decades and many studies on the hydrogeochemistry of pit lakes are currently available (e.g. Davis and Ashenberg 1989, Karakas et al. 2003, Sánchez-España et al. 2008a, 2008b, 2009, 2014a, 2014b, Geller et al. 2013). In Chapter 1, the different stratification patterns typical of mine pit lakes (holomictic vs. meromictic) were described. The majority of the pit lakes in the Iberian Pyrite Belt (IPB) are meromictic (Sánchez-España et al. 2008a). The water column is divided in two layers, the mixolimnion (upper layer, oxic) and the monimolimnion (bottom layer, anoxic), separated by an intermediate and transitional layer, the chemocline, characterized by sharp physico-chemical gradients. During the warmer months, the mixolimnion of meromictic lakes develops two sub-layers, the epilimnion and hypolimnion, although in some of them these two sub-layers are present also during the coldest months.

Cueva de la Mora reached hydrological equilibrium after 1975, and the deep part of the lake appears to have been hydrologically isolated from the local groundwater flow since then (Sánchez-España et al. 2014a). On the other hand, Guadiana is still being filled by groundwater input at 55 m depth, and it has been calculated that this lake will reach hydrological equilibrium around 2018 (Sánchez-España et al. 2014b). The geochemistry of these two lakes has been described in recent publications by Sánchez-España et al. (2008a, 2009, 2013, 2014a, 2014b). The Cueva de la Mora pit lake has an elongated shape and its maximum depth is approximately 38-40 m (Fig. 2.1), depending on the annual rainfall regime, and its surface area is 1.79 ha. The Guadiana pit lake shows a funnel shape (Fig. 2.2), it is currently about 62 m deep and still being filled with ground- and surface-waters (Sánchez-España et al. 2014b). This lake has surface area of 1.71 ha.

In previous works, the mixolimnion in Cueva de la Mora has been reported to extend to a depth of 8-10 m, and to have a pH of between 2.3 and 3.1 (Diez-Ercilla et al. 2014), while this water layer in the Guadiana pit lake is approximately 20 m deep and is itself stratified, with an upper oxic epilimnion overlying an anoxic hypolimnion (Falagán et al. 2014, Sánchez-España et al. 2014b). The chemocline occurs as a relatively narrow (2 m thick) band in Cueva de la Mora, while in Guadiana it is not well defined. The monimolimnion in the Cueva de la Mora pit lake (pH 4.0 to 4.5) has been reported to have several sub-layers due to the influence of lateral inflows of water from connecting

galleries from deep mining works (Fig. 2.1) (Sánchez-España et al. 2009). Similarly, intersecting galleries (Fig. 2.2) at different depths also influence the monimolimnion of the Guadiana pit lake (pH 3.9 to 4.2), and there is evidence that some alkaline waters enter this pit lake through its lower water layer, which is characterized by the presence of high amounts of CO₂ (Sánchez-España et al. 2014b). Iron concentrations and speciation vary with depth in both lakes. Virtually all of the soluble iron in the mixolimnion zones is ferric, while in the monimolimnion zones it is almost exclusively ferrous iron. Total concentrations of soluble iron in the mixolimnetic layers are between one and two orders of magnitude lower than those in the monimolimnion (Sánchez-España et al. 2009).

The present chapter provides an outlook of the main physico-chemical parameters (e.g. pH, temperature, conductivity, redox, dissolved oxygen, metal and nutrient concentration) of the pit lakes that have been selected for the microbiological investigation. The work includes data from different field campaigns conducted between 2010 and 2012 during the course of the present study. These data constitute the geochemical basis for the microbiological results presented in the following chapters.

2.2 MATERIAL AND METHODS

2.2.1 STUDY SITES AND SAMPLING TECHNIQUES

The two pit lakes studied were Cueva de la Mora (37°36'07"N, 7°17'84"W) and Guadiana (Herrerías mine; 37°46'66"N, 6°49'31"W). Redox potentials (E_H), pH values, temperatures (T), specific conductance (SC) and dissolved oxygen (DO) were measured using Datasonde DS5 and MS5 probes (Hydrolab® Hach Company, Colorado, USA) between 2010 and 2012. Water samples were taken from both pit lakes during three field campaigns (September 2011 and March and September 2012), and sediments were sampled on one occasion (February 2012). Water samples were taken from the different stratified zones in the two pit lakes using a 5 L Van Dorn sampler (KC-Denmark). In September 2011, water samples were obtained at Cueva de la Mora at 3, 8.5 and 35 m depths, and in Guadiana at 7, 20 and 50 m. In March and September 2012, water samples were obtained at Cueva de la Mora at 3, 10.5, 15.2 and 35 m depths, and in Guadiana at 4.6, 7, 15, 20 and 50 m. Water samples (500-1000 mL) were filtered through sterile 0.2 µm (pore size) nitrocellulose membranes and N-free membranes (for nitrate

and ammonium determination analyses). Filtered water was acidified with HCl and stored at 4°C for subsequent chemical analysis.

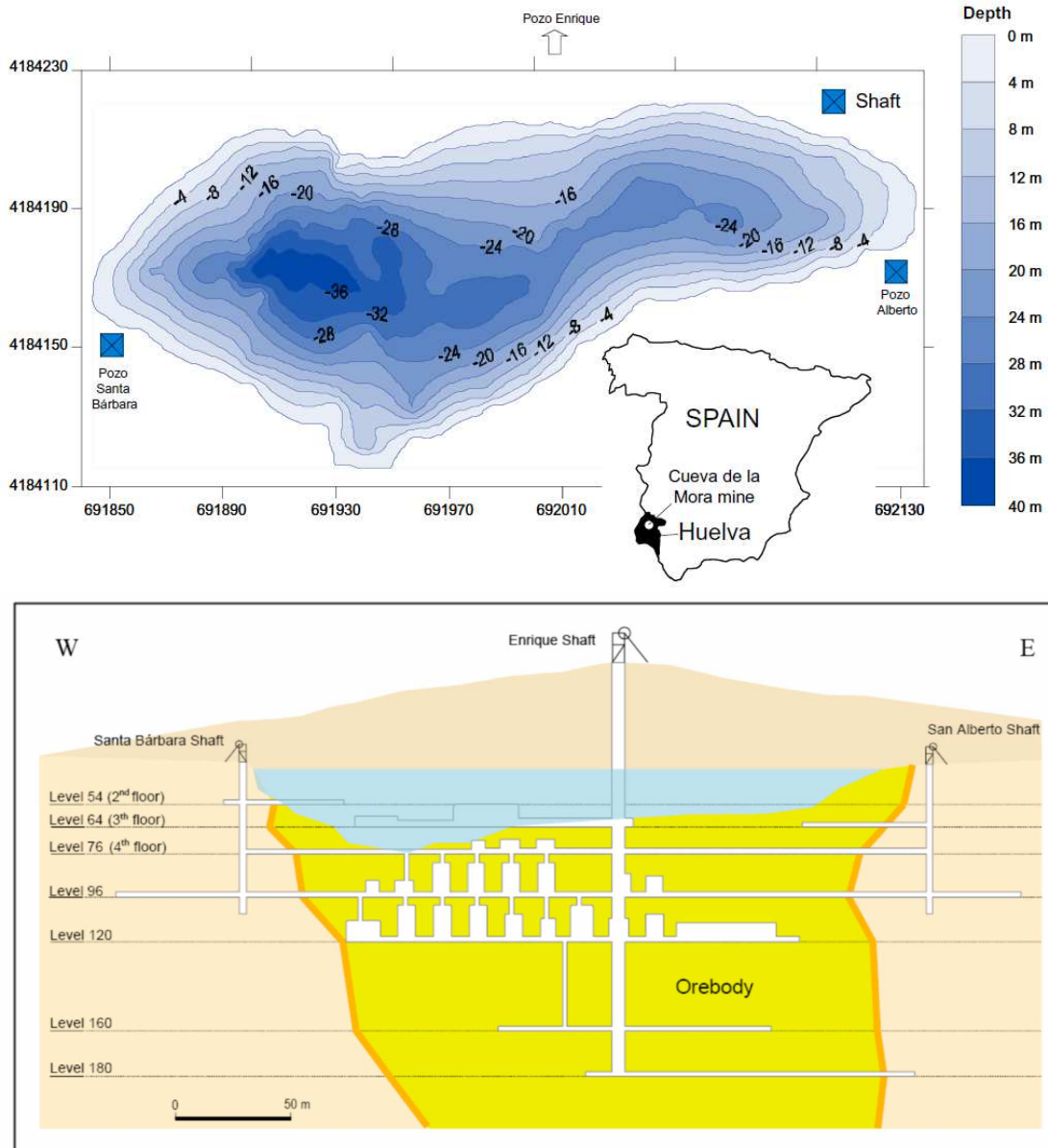


Figure 2.1. Bathymetry of the Cueva de la Mora pit lake (top) and representation of the open cast and mine galleries (bottom) of the Cueva de la Mora mine (reprinted from Sánchez-España et al. 2014a).

Sediment samples in Cueva de la Mora were obtained from the monimolimnetic zone (38 m depth) and from a shelf in the mixolimnetic zone (8 m depth) using an Uwitec® gravity corer (Uwitec, Mondsee, Austria). Cores, 8.6 cm diameter and 12 cm

(mixolimnion sample) and 17 cm (monimolimnion sample) in length, were sealed and stored at 4°C until they were processed. After a week they were sectioned vertically at ~2 cm intervals. Interstitial water was extracted to analyse pH and E_H by centrifuging at 5,400 g for 10 minutes. Sediments used for chemical analysis were stored at 4°C. The recovery of a continuous core from the deep part of the Guadiana pit lake was impossible as the high dissolved gas content (Sánchez-España et al. 2014b) caused the sediment to mix when raised to the surface breaking the stratigraphy.

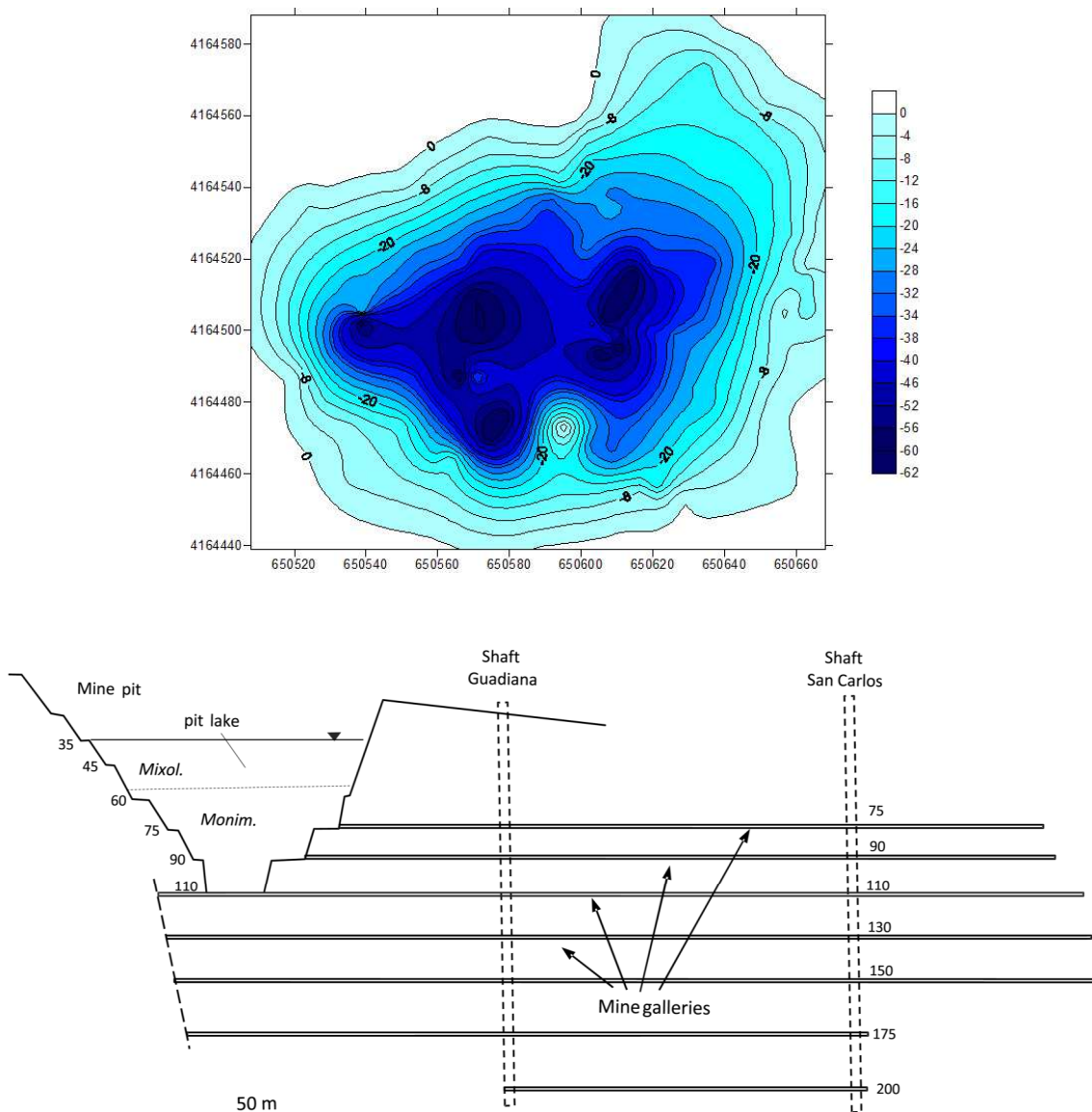


Figure 2.2. Bathymetry (top) of the Guadiana pit lake and idealized section of the open cast and mine galleries of the Herrerías mine at the Guadiana site (bottom; reprinted from Sánchez-España et al. 2014b).

2.2.2 LABORATORY-BASED CHEMICAL ANALYSES

Analysis of ammonium- and nitrate-nitrogen and phosphate from water samples were carried out soon (2-6 hours) after sampling. Water analyses from the sampled depths were carried out with a portable UV-VIS DR2800 spectrophotometer (Hach) and Lange cuvette tests. The methods used were LCK 138 (1-16 mg/L Total Nitrogen, TN), LCK 349 (0.05-1.50 mg/L P-PO₄³⁻), LCK 385 (3-30 mg/L, total organic carbon, TOC), LCK 304 (0.02-2.0 mg/L N-NH₄⁺). The methods LCK 138, LCK 349 and LCK 358 required a previous digestion with a Hach-Lange LT100 thermostat, and in the case of the LCK 385, samples were shaken 5 minutes with a Hach-Lange TOC-X5 shaker.

Nitrate was measured with an ion-selective electrode Nitrate ionplus[®] Sure-flow[®] and reference electrode 9307BNWP coupled to an Orion Start[™] meter (Thermo Fisher Scientific Inc.). Calibration was performed with different standards prepared from a NO₃⁻ standard (1,000 mg/L) supplied by the company.

Off-site analysis (September 2012 samples only) of sulphate, iron, dissolved organic carbon (DOC), transition metals and aluminium were carried out 2-4 days after the sampling in the facilities of BART (*Bangor Acidophile Research Team*) at Bangor University (Wales, UK). The samples were cold stored. Aluminium was determined by atomic absorption spectrophotometry using a Varian Spectr AA 220 FS. Sulphate was measured using a Dionex IC25 ion chromatograph with an Ion Pac AS-11 column equipped with a conductivity detector. Transition metals were measured with a Dionex-320 ion chromatograph fitted with an IonPAC[®] CS5A column and an AD absorbance detector. Concentrations of total DOC cell-free water samples were measured using a LABTOC DOC analyser (Pollution and Process Monitoring, UK). The rest of the analytical work was done at the IGME laboratories (Tres Cantos, Madrid).

2.3 RESULTS

The physico-chemical profiles and chemical parameters of the water columns reported in the present study (Table 2.1, and Fig. 2.3 to Fig. 2.5) are similar to those previously reported for both pit lakes (Sánchez-España et al. 2009, 2013, 2014a, and 2014b).

2.3.1 WATER COLUMN CHEMISTRY

The upper 8 m of the Cueva de la Mora pit lake (the mixolimnion) are aerobic, while the water column below 10 m (the monimolimnion) is essentially devoid of oxygen and presents sub-layers reflected on the specific conductivity (Fig. 2.3). The transition zone between these two layers (the chemocline) is characterized by steep gradients in pH, E_H and DO concentrations (Figs. 2.3 and 2.4). In September 2012, a DO maximum was found just above the chemocline, while in March 2012 there was no corresponding DO peak. The concentrations of dissolved ionic solutes were, as anticipated, uniform in the mixolimnion but increased with depth through the chemocline and monimolimnion zones. Redox potentials and pH values in the water column (Fig. 2.4) followed similar trends to those reported by Sánchez-España et al. (2009, 2013). The temperature of the upper 10 m of Cueva de la Mora is influenced by seasonal fluctuations (10°C in March vs. 25°C in September 2012) unlike water at lower depths (Fig. 2.5).

The Guadiana pit lake shows a mixolimnion that, as noted previously, could be subdivided into two layers: an epilimnion (0-8 m depth), which is aerobic (Fig. 2.3) and influenced by seasonal temperature changes (15°C in March to 25°C in September 2012), overlying an anoxic hypolimnion (8-20 m depth), within which all measured physico-chemical parameters showed no depth-related changes due to physical mixing of water within this zone. The SC profile follows an increasing pattern up to 20 m, but a very marked increase is clearly evidenced in the monimolimnion and stabilizes at 50 m up to the bottom of the lake (Fig. 2.3). This pit lake displays a more complex redox potential profile than that of Cueva de la Mora (Fig. 2.4). The upper 10 metres are influenced by seasonality but for the next 10 metres, the redox potential remains constant and then decreases rapidly (~+440 mV) up to 25 m where presents a slight decrease up to the bottom (Fig. 2.4). The profile for temperature also shows variations in the upper ~12 m due to seasonality with a rapid decrease down to 15°C between 15 and 20 m. Down to the bottom temperature shows a continuous increase with depth to a value of 26°C (Fig. 2.5). The pH is lower (2.0-3.0) between 10 and 20 metres than in the upper meters of the water column (2.7-3.2), but then increases rapidly to 4.0-4.5 in the chemocline and remains relatively constant in the monimolimnion (Fig. 2.4). The monimolimnion appears to be divided in two sub-layers, all the physico-chemical parameters described above appear to follow an increasing or decreasing pattern up to a depth of 50 m where 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. (2014a).

The main redox couple in the water column of both lakes is ferrous/ferric iron. Ferric iron is the dominant form of soluble iron in the mixolimnion of Cueva de la Mora and in the epilimnion of Guadiana, while ferrous iron accounted for ~100% of the soluble iron in the monimolimnion layers of both pit lakes. The chemocline zone (Cueva de la Mora) and the hypolimnion (Guadiana) contained both ferrous and ferric iron (Table 2.1). This fact has important implications with regard to the microbial ecology of these lakes, as will be discussed in following chapters. Sulphate is very abundant in both lakes, in the monimolimnion the concentration is one magnitude order larger than that in the upper layer. Both mixolimnions are scarce in phosphate (PO_4^{3-}), ammonium (NH_4^+) and nitrate (NO_3^-). The monimolimnion of Cueva de la Mora bottom layer phosphate concentration is ~7 mg/L (Table 2.1).

Table 2.1. Physicochemical parameters of water samples from different depths within the Cueva de la Mora and Guadiana pit lakes. *calculated from E_H data and total iron concentrations, using the Nernst equation; **analysed in September 2011; †, Sánchez-España personal communication; <, not detectable.

Pit lake	Depth (m)	pH	E_H (mV)	Fe_{total} mg/L	Fe^{2+*} mg/L	DOC mg/L	$\text{SO}_4^{2-}\text{-S}$ mg/L	$\text{PO}_4^{3-}\text{-P}^{**}$ mg/L	$\text{NH}_4^+ + \text{NO}_3^-\text{-N}^{**}$ mg/L
Cueva de la Mora	6	2.6	+844	546	2.9	1.8	620	0.026	1.003
	10.5	3.0	+577	752	748	2.9	1215	0.065	0.872
	30	4.5	+304	4910	4910	5.3	3990	6.98	1.340
Guadiana	7	2.9	+765	45	4.7	1.04	870	<	0.141
	15	2.7	+697	205	128	4.01	1355	<	0.124
	55	4.2	+333	7600	7600	8.41	7405	0.226	0.484
San Telmo†	5	2.8	+869	153	0.8	n.a.	n.a.	<	<
	25	2.7	+879	197	1.2	n.a.	n.a.	<	<
	90	2.8	+680	212	146	n.a.	n.a.	0.161	0.039

The most abundant metallic elements in the lake waters, after iron, were aluminium and magnesium (Tables 2.1 and 2.2). The lake bottom waters usually contained much higher concentrations of more metals and metalloids with respect to the upper layers, except for aluminium in the Cueva de la Mora lake, whose concentration decreased with

depth as a result of the precipitation of aluminium hydroxysulphates at pH >4.0 (Sánchez-España et al. 2011).

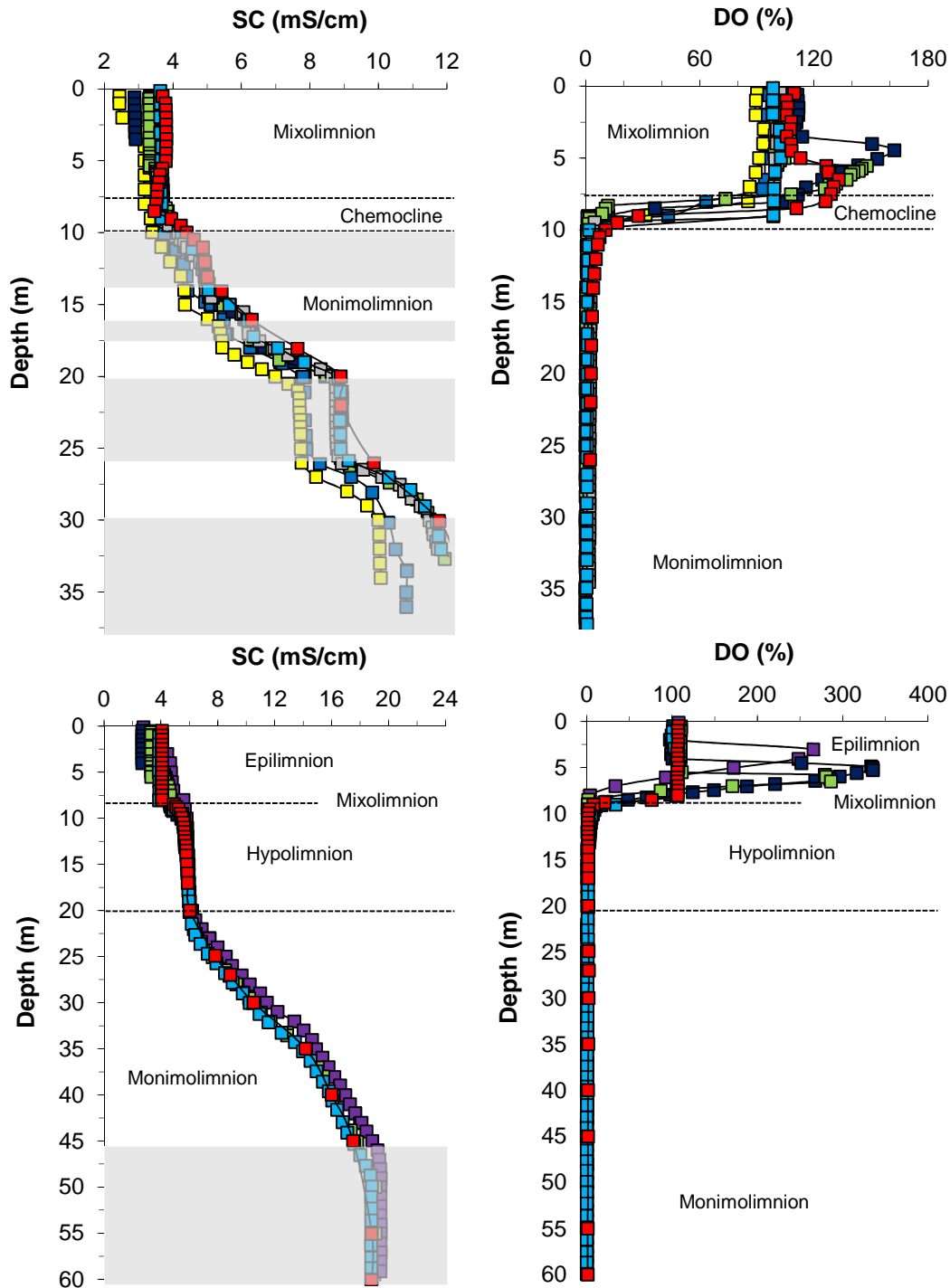


Figure 2.3. Stratification and depth-related profiles of specific conductivity (SC, mS/cm) and dissolved oxygen (DO, %) in Cueva de la Mora (top) and Guadiana (bottom) pit lakes. ■, November 2010; ■, March 2011; ■, April 2011; ■, July 2011; ■, September 2011; ■, March 2012; ■, September 2012.

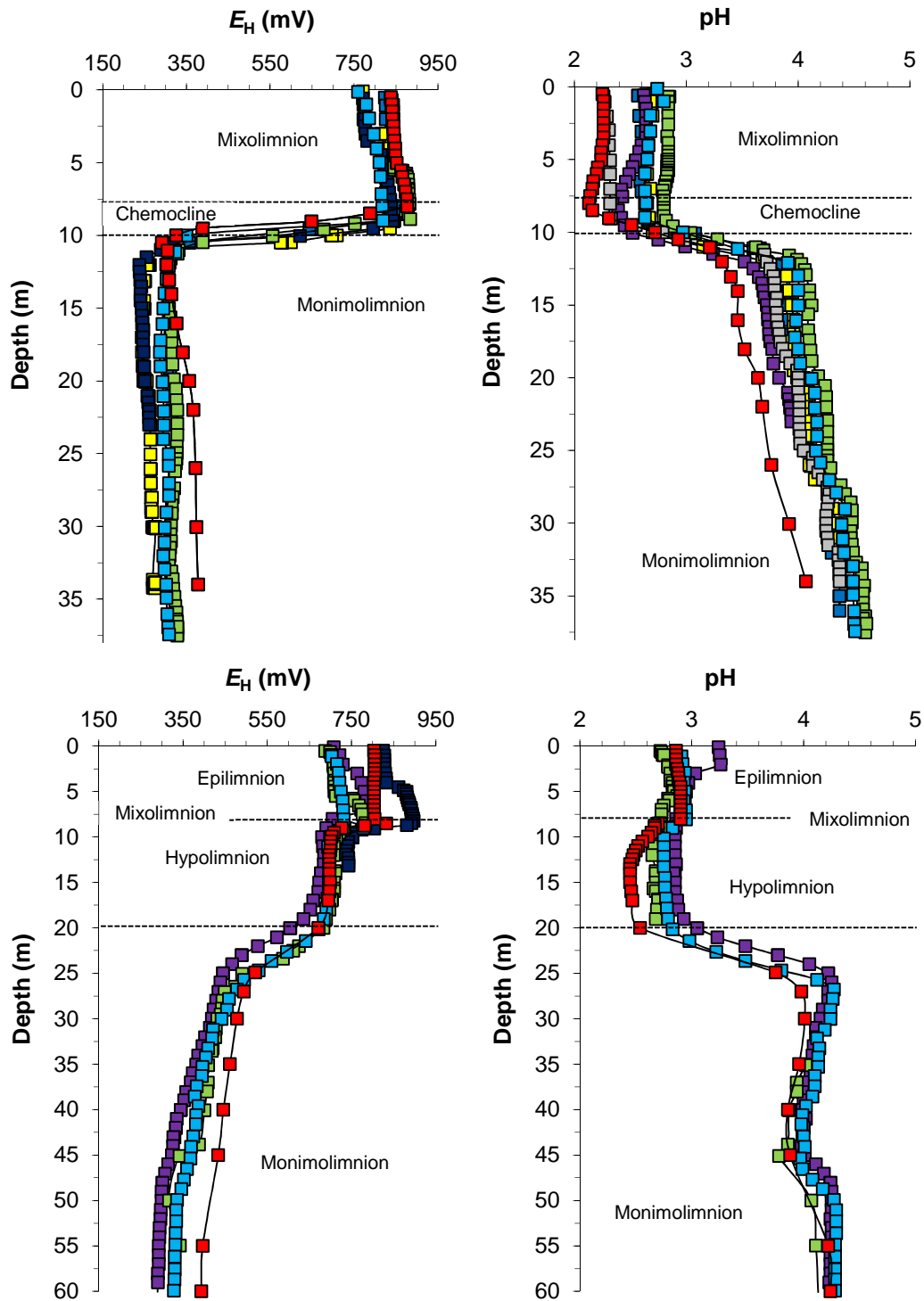


Figure 2.4. Stratification and depth-related profiles of redox potentials (E_H) and pH values in Cueva de la Mora (top) and Guadiana (bottom) pit lakes. ■, November 2010; ■, March 2011; ■, April 2011; ■, July 2011; ■, September 2011; ■, March 2012; ■, September 2012.

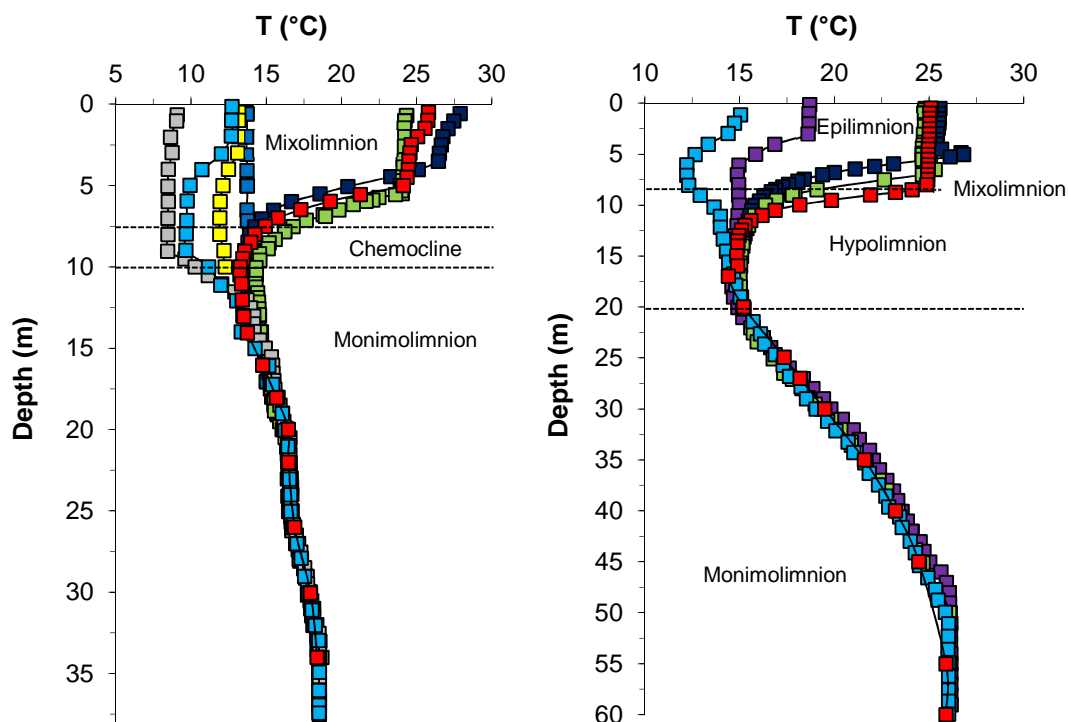


Figure 2.5. Temperature of the water column in Cueva de la Mora (top) and Guadiana (bottom) pit lakes.

■, November 2010; ■, March 2011; ■, April 2011; ■, July 2011; ■, September 2011; ■, March 2012; ■, September 2012.

Table 2.2. Metal concentrations of water samples from different depths of Cueva de la Mora and Guadiana pit lakes. *samples analysed in July 2011 (Cueva de la Mora) and in September 2011 (Guadiana); +Sánchez-España personal communication.

Pit lake	Depth (m)	Al	Mn	Zn	Mg*	As*	Cu*	Co*	Ni*
		mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Cueva de la Mora	6	109	17	17	134	0.045	3.9	0.8	0.4
	10.5	126	30	31	181	0.092	1.7	1.2	0.5
	30	70	110	95	3520	14.650	0.03	2.1	0.7
Guadiana	7	71	17	68	348	0.021	21	1.8	1.4
	15	108	118	109	490	0.240	26	2.3	1.7
	55	151	394	305	2707	0.170	1	9.7	6.3
San Telmo+	5	120	31	65	388	0.055	17	0.7	0.4
	25	161	51	101	596	0.072	26	1.1	0.5
	90	129	67	120	672	0.028	28	1.4	0.6

2.3.2 SEDIMENT CHEMISTRY

Some important physico-chemical differences were noted between sediments taken at 8 m depth (“shore sediments”) and those taken at 38 m depth (“deep basin sediments”) in the Cueva de la Mora pit lake (Fig. 2.6). The pH of the shore sediments decreased from 3.0 at the sediment-water interface to 2.6, at 12 cm, while redox potentials ($\sim +300$ mV) showed slight depth-related changes (Fig. 2.7). The basin sediments had higher pH values (which increased with distance from the sediment surface from 3.9 to 4.3) and lower redox potentials (which decreased with depth from +193 mV to +160 mV).



Figure 2.6. Photographs of the core sediment samples obtained from the Cueva de la Mora shelf shore (left) and deep basin (right).

Sediments were more rich in organic carbon and phosphate content than the water column. Also, there are differences between sediments at both depths, with organic carbon and phosphate content being higher in the shore sediment sample (~ 150 mg/L and ~ 3.3 mg/L respectively), than those in the deep basin sediment sample (~ 50 mg/L and 0.34 mg/L respectively).

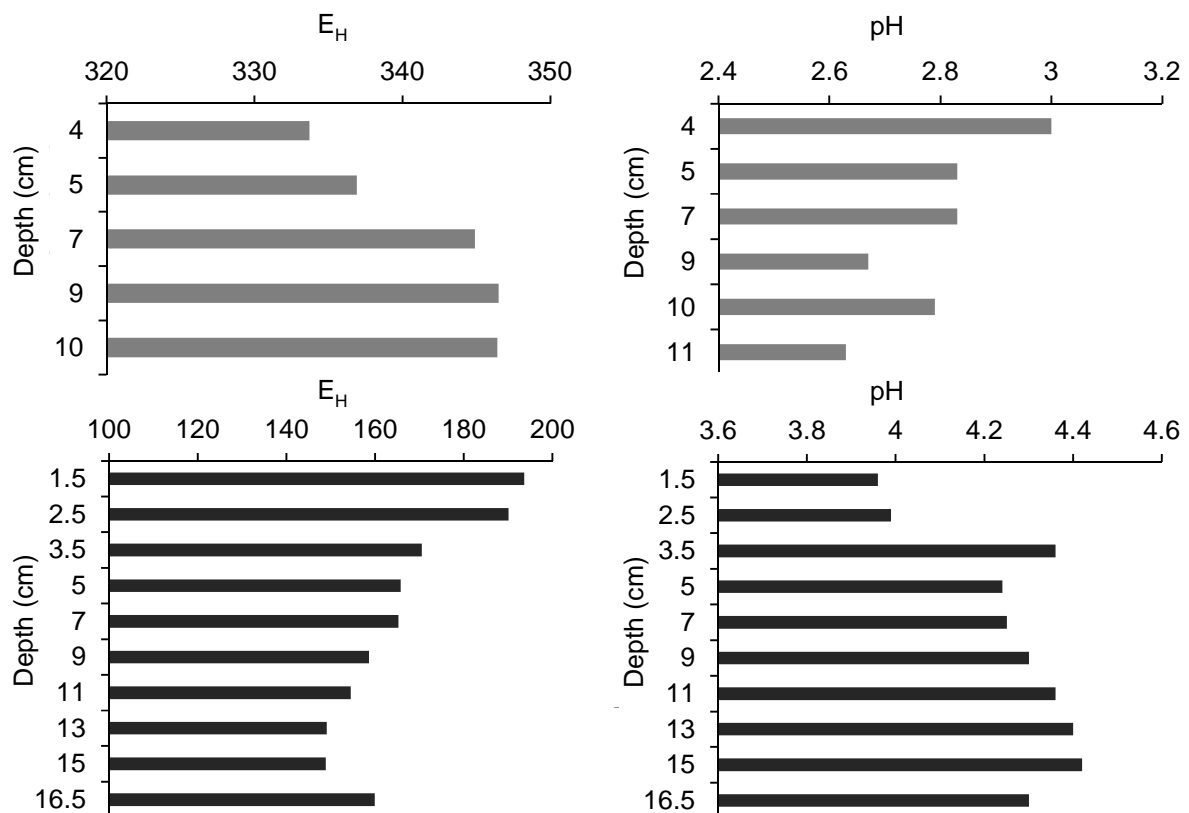


Figure 2.7. pH and redox potential values in shore sediments (top) and deep basin sediments (bottom) of the Cueva de la Mora pit lake.

2.4 DISCUSSION

The Cueva de la Mora and Guadiana pit lakes were chosen among many others in the IPB because they display the most dramatic vertical gradients of pH and water chemistry of those reported to date. These lakes therefore represent world-class examples of meromictic pit lake stratification with a complex (sub-layered) internal structure of their anoxic water bodies. The stratification patterns found in the two pit lakes studied revealed the co-existence of three “environments” with highly contrasting physico-chemical characteristics within the same water body. These three zones (the mixolimnion, chemocline and monimolimnion in Cueva de la Mora, and the epilimnion, hypolimnion and monimolimnion in Guadiana) are interconnected by a vertical movement of metal oxyhydroxide precipitates (downwards flux of oxidized species) and

ionic diffusion (upwards flux of reduced species and gaseous diffusion at the lake/atmosphere interface).

Although these two pit lakes could appear to be similar, there are several differences between them, which are worth discussing. Firstly, Cueva de la Mora has already reached its maximum capacity and its water level remains constant, although some water level changes are caused by evaporation and rainfall over the year. On the other hand, the Guadiana pit lake is still being filled as stated by Sánchez-España et al. (2014b) and also observed during serial samplings. Secondly, the water column of these pit lakes presents significant different characteristics. Cueva de la Mora mixolimnion and Guadiana epilimnion are under the influence of varying weather conditions, so that physico-chemical parameters such as temperature and SC vary seasonally as a result of changing solar radiation intensity and rainfall regime, respectively. It is the transition layer in Cueva de la Mora (the chemocline) where these parameters change rapidly to give way to the monimolimnion where all the physicochemical features remain rather constant. However, looking closely at the conductivity values it is possible to identify sub-layers caused by connections to the underground tunnels from the former mine. In Guadiana, the chemical transition layer is situated below the bottom of the hypolimnion at 20 m depth where the monimolimnion starts. This bottom layer is rather homogeneous and shows smooth but continuous gradients of T, SC and E_H . However, in the last 15-10 m of the water column these three parameters stabilize and remain constant. This might be caused by the inflow of groundwater existing at 55 m (Sánchez-España et al. 2014b) which causes a homogenization of the water at that depth. Finally, the monimolimnion of the Guadiana pit lake is over saturated on CO_2 , which characterizes this lake as a possible threat to the surrounding populations in the hypothetical case of gas eruption (Sánchez-España et al. 2014b). This, however, will not be discussed in the present study.

Other mine pit lakes in the IPB present less complex physico-chemical profiles. For example, the San Telmo pit lake is the deepest pit lake in the IPB (~130 m, Sánchez-España et al. 2013) and also meromictic, but its monimolimnion is completely homogeneous and its chemocline is located at 29 m (Fig. 2.8). As in the other lakes, the mixolimnion is oxygen-rich and presents seasonal stratification from early spring to late autumn (epilimnion, upper warm layer; metalimnion, transition layer; and hypolimnion, lowest and coldest layer), (Sánchez-España et al. 2007a). There are other examples of meromictic lakes with homogeneous monimolimnion in the IPB such as Confesionarios

(López-Pamo et al. 2009). Moreover, other pit lakes in the IPB present other stratification patterns. For example, Nuestra Señora del Carmen is considered a holomictic lake presenting periods of meromixis during the cold months (Santofimia et al. 2012). The Concepción pit lake is a relatively shallow (16 m deep) meromictic lake where the inflow of groundwater and runoff causes the development of different layers normally present in deepest water masses (Santofimia and López-Pamo 2013).

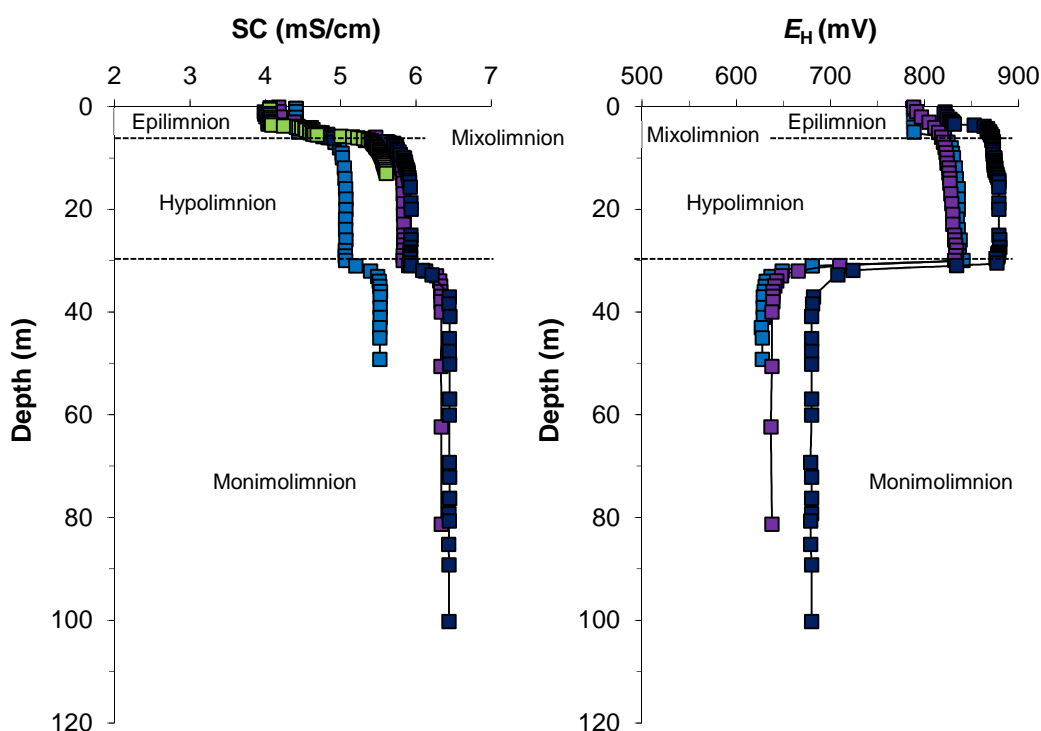


Figure 2.8. Specific conductivity (SC) and redox potential (E_H) of the San Telmo pit lake. ■, November 2010; ■, April 2011; ■, July 2011; ■, September 2011; (Sánchez-España personal communication).

As many other acid mine waters, the Cueva de la Mora and Guadiana pit lakes are notably rich in sulphate and dissolved metals. Metal concentrations increase with depth (Table 2.2) and, even though there are two remarkable exceptions to this assumption, copper and aluminium decrease. Copper reacts with the biologically produced H_2S present in the Cueva de la Mora transitional layer forming copper sulphides in the chemocline (Diez-Ercilla et al. 2014, Falagán et al. 2014). Aluminium forms Al-hydroxysulphates at the Cueva de la Mora lake bottom as the pH increases (Sánchez-España et al. 2011). It is well known that the chemistry of the majority of pit lakes in the

IPB is controlled by the iron buffer system at pH 2.2-3.5, while at pH >4 (usually at the molimnion) the water chemistry is controlled by the aluminium buffer system (Sánchez-España et al. 2011). This is not the case of San Telmo pit lake, where the pH of the whole water column is always <3, therefore, is only controlled by the iron buffer system (Table 2.1). In the San Telmo pit lake, metallic elements are in similar concentrations in the whole water column than those in both Cueva de la Mora and Guadiana lakes, except for those in the monimolimnion (especially Fe but also Mg or Mn), which are significantly lower in San Telmo than in the other two pit lakes.

The presence of dissolved metals in the water column and the nutrient concentrations in the water column of Cueva de la Mora and Guadiana lakes, may have important consequences with regard to the microbial communities inhabiting these extreme environments (Table 2.1 and 2.2). Elevated concentrations of soluble metals usually have negative effects on microorganisms. Moreover, it is well known that nutrients availability is the limiting factor for the growth of organisms. The Cueva de la Mora and Guadiana waters are rich in dissolved metals and poor in nutrients (nitrogen and phosphorus). The effects of low bioavailability of nutrients and the presence of metals in the lake waters and how these two factors may influence the microbial community inhabiting them will be discussed in the following chapters, as it is thought that these are determining factors on the development of the lake waters.

Finally, while sediments in rivers and streams of the IBP have been studied before (e.g. Rowe et al. 2007, Sánchez-Andrea et al. 2011) there have been relatively few reports describing the microbial diversity of sediments in metal-mine pit lakes in any region of the world. The sediment analysed and described in this work present different physico-chemical features than those of the water column at the same depth, this is especially true as regards the pH and nutrient concentrations, which are both slightly higher. In addition, the redox potential reflects the oxygen depletion in the mixolimnetic sediments with increasing depth from the water/sediment interface, as well as in the whole thickness of the monimolimnetic sediments. Moreover, the vertical variations of pH and redox potential within the sediment samples (Fig. 2.7) provides evidence for the existence of different environments through the length of the sediment sample. The study of the microbial populations present in both sediments (shore and deep-basin sediments) will be discussed in the following chapters.

2.5 CONCLUSIONS

The Cueva de la Mora and Guadiana pit lakes represent exceptional examples of meromictic stratification with vertical gradients of water chemistry and redox conditions, which make them excellent laboratories for the study of geomicrobiological processes and geomicrobial interactions in extremely acid and metal-laden environments.

The Cueva de la Mora water column presents a very specific stratification pattern caused by underground connections with the mine tunnels. The stratification pattern of the Guadiana water column is influenced by climatology at the shallower layer and also by groundwater inflow at the bottom of the lake.

Sediments in the Cueva de la Mora pit lake present different physico-chemical features depending on the depth (shore vs. deep basin sediments) and the geochemistry of its pore waters can substantially vary from that of the overlying water column.

These characteristic stratification patterns coupled with the high metal concentrations and nutrient distributions in the lake waters make them very distinct environments for the development of microbial communities.

**MICROBIOLOGY OF
CUEVA DE LA MORA AND
GUADIANA PIT LAKES**

3

The present chapter includes the main findings and results published in the paper by Falagán et al. (2014): **New insights into the biogeochemistry of extremely acidic environments revealed by a combined cultivation-based and culture-independent study of two stratified pit lakes. *FEMS Microbiol Ecol* 87:231-243.**

3.1 INTRODUCTION.....	56
3.2 MATERIAL AND METHODS.....	57
3.2.1 SAMPLING TECHNIQUES AND SAMPLING DATES.....	57
3.2.2 PIGMENT ANALYSES.....	58
3.2.3 DETERMINATION OF VIABLE COUNTS OF MICROORGANISMS AND ISOLATION OF ACIDOPHILES.....	58
3.2.4 BIOMOLECULAR ANALYSES.....	60
3.3 RESULTS.....	63
3.3.1 WATER MICROBIOLOGY.....	63
3.3.1.1 PHOTOTROPHIC COMMUNITY.....	63
3.3.1.2 PROKARYOTIC COMPOSITION.....	69
3.3.1.2.1 CUEVA DE LA MORA.....	70
3.3.1.2.2 GUADIANA PIT LAKE.....	75
3.3.2 SEDIMENT MICROBIOLOGY: CUEVA DE LA MORA.....	76
3.3.3 IDENTIFICATION AND CHARACTERISTICS OF ACIDOPHILIC BACTERIA ISOLATED FROM THE CUEVA DE LA MORA AND GUADIANA PIT LAKES.....	78
3.4 DISCUSSION.....	78
3.5 CONCLUSIONS.....	87
3.6 APPENDIX.....	89

Abstract

The indigenous microbial communities of two extremely acidic, metal-rich stratified pit lakes, located in the Iberian Pyrite Belt (Spain) were identified, and their roles in mediating transformations of carbon, iron and sulphur confirmed. A combined cultivation-based and culture-independent approach was used to elucidate microbial communities at different depths and to examine the physiologies of isolates, which included representatives of at least one novel genus and several species of acidophilic bacteria. Phosphate availability correlated with redox transformations of iron, and this (rather than solar radiation) dictated where primary production was concentrated. Carbon is fixed and released as organic compounds by acidophilic phototrophs acted as electron donors for acidophilic heterotrophic prokaryotes, many of which catalysed the dissimilatory reduction of ferric iron; the ferrous iron generated was re-oxidized by chemolithotrophic acidophiles. Bacteria that catalyse redox transformations of sulphur were also identified, though these appeared to be less abundant than the iron oxidizers/reducers. Primary production and microbial numbers were greatest and biogeochemical transformation of carbon, iron and sulphur most intense, within a zone of ~8-10 m depth, in the transition layer, in both pit lakes. Archaea detected in sediments included two *Thaumarchaeota* clones, indicating that members of this recently-described phylum can inhabit extremely acidic environments.

3.1 INTRODUCTION

The Iberian Pyrite Belt (IPB) represents the world's largest accumulation of massive sulphide deposits and has been mined from pre-Roman times to the present day for precious (Au, Ag) and base (Cu, Pb, Zn) metals, and sulphur (Leistel et al. 1998). Most of the mines were abandoned during the twentieth century leaving an extensive legacy of waste rock dumps, tailings impoundments and flooded opencast pits. When active mining ends, opencast voids become filled with ground- and surface-waters. Exposure of residual sulphide minerals to both oxygen and water results in their oxidative dissolution and the subsequent formation of metal-rich pit lakes, which are often extremely acidic (Geller et al. 1998, 2013, Sánchez-España et al. 2008a).

Some acidophilic bacteria and archaea are able to accelerate the rate of pyrite oxidation by several orders of magnitude compared with abiotic (chemical) dissolution under the same conditions of temperature, pH, etc. (Singer and Stumm 1970, Nordstrom and Southam 1997, Nordstrom and Alpers 1999). Many studies have focused on the geochemistry and microbiology of acid mine drainage (AMD) affected streams and other mine-impacted water bodies, not only because these environments are a major source of environmental pollution, but also because microorganisms indigenous to these environments are used in commercial bio-processing of ores and concentrates and have potential for bioremediating acidic environments (Johnson 2013). Most of these studies have been carried out with AMD streams (e.g. Baker et al. 2004, Rowe et al. 2007), and acidic rivers (e.g. López-Archilla and Amils 1999, López-Archilla et al. 2000). Some studies have focused on pit lakes (e.g. in Germany, USA, Australia and China), but relatively few of these have been located in the IPB where more than twenty such water bodies have been identified (Sánchez-España et al. 2007a, Wendt-Potthoff et al. 2012, Santofimia et al. 2013). Pit lakes in the IPB are often very diverse in size, depth, age and water composition, though they share a common geological framework. Most of those described are meromictic and display a permanent stratification pattern, the water column being composed of an upper oxic layer (the mixolimnion), a bottom anaerobic layer (the monimolimnion) and a transitional chemocline separating the two zones (Sánchez-España et al. 2009, 2013). Therefore, pit lakes differ in many fundamental ways from AMD streams, which are not stratified, though the latter change in their physico-chemical and microbiological characteristics with distance from the source of the AMD discharge (e.g. Walton and Johnson 1992).

The present study focused on isolating and characterizing acidophilic microorganisms from the Cueva de la Mora and Guadiana pit lakes, and investigating the relationships between the indigenous microbial communities and the contrasting physico-chemical characteristics of the stratified water layers. The role of microorganisms in mediating the transformation and cycling of carbon, iron and sulphur, previously observed in the water columns of these lakes, and to the identities of those involved, were also key objectives of the study.

3.2 MATERIAL AND METHODS

3.2.1 SAMPLING TECHNIQUES AND SAMPLING DATES

Photosynthetically-active radiation (PAR), dissolved oxygen (DO) and chlorophyll-a were measured using Datasonde DS5 and MS5 probes (Hydrolab® Hach Company, Colorado, USA) during field trips conducted during the years 2010 to 2012. Samples were taken from the water column using a 5 L Van Dorn sampler (KC-Denmark) in both pit lakes during four field campaigns (namely in July and September 2011, and in March and September 2012), and sediments were sampled on one occasion (February 2012). For pigment analysis water samples were taken at 2, 4.2, 5.2, 8 and 12.3 m in Cueva de la Mora and at 1.5, 4.5, 8.2 and 12 m in Guadiana in July and September 2011. Water samples for microbiological analysis were taken from the different stratified zones in the two pit lakes. These three depths were chosen as representative of the mixolimnion, chemocline and monimolimnion of both lakes. In September 2011 samples were collected at Cueva de la Mora at 3, 8.5, and 35 m depths, and in Guadiana at 7, 20 and 50 m. In March 2012, water samples were obtained at Cueva de la Mora at 3, 10.5, 15.2 and 35 m depths and in Guadiana at 4.6, 7, 15, 20 and 50 m. In September 2012, samples were taken in Cueva de la Mora at 6, 10.5 and 30 m and in Guadiana at 7, 15 and 55 m. Water samples (500-1000 mL) were filtered through sterile 0.2 µm (pore size) nitrocellulose membranes, which together with the microbial biomass collected on them, were stored at -20°C prior to extraction of DNA. Unfiltered water samples, used for the most probable number technique (MPN) and for isolating bacteria on solid media, were stored at 4°C.

Sediment samples in Cueva de la Mora were obtained from the monimolimnetic zone (38 m depth) and from a shelf in the mixolimnetic zone (8 m depth) using an Uwitec® gravity corer (Uwitec, Mondsee, Austria). Cores, 8.6 cm diameter, and 12 cm

(mixolimnion sample) and 17 cm (monimolimnion sample) in length, were sealed and stored at 4°C until they were processed. After one week, they were sectioned vertically at ~2 cm intervals. Sediments used for isolation of microorganisms were stored at 4°C, and those used for DNA extraction were frozen at -20°C.

3.2.2 PIGMENT ANALYSES

Water lake samples (1-2 L) were filtered through 0.7 µm glass fibre filters GF/F Whatman and stored at -20°C. Pigment extraction was performed at room temperature. Biomass containing glass fibre filters were washed with neutralized acetone to avoid pigment reduction. Neutralization was achieved by passing the acetone throughout MgCO₃. Biomass containing acetone was stored at 4°C in total darkness for 24 hours until pigment extraction. Pigment composition was determined in the Faculty of Marine Sciences and Environmental Sciences of the University of Cádiz (Department of Biology, Puerto Real, Spain) and in the Helmholtz Environmental Research Centre-UFZ (Magdeburg, Germany) with spectrophotometers scanning at absorbance of 300 to 900 nm.

3.2.3 DETERMINATION OF VIABLE COUNTS OF MICROORGANISMS AND ISOLATION OF ACIDOPHILES

Two different techniques were performed to quantify microbial cells in the pit lake waters: MPN (Most Probable Number; indirect technique) and colony-forming units (direct technique).

MPN consists in the cultivation of samples with different specific culture media in 96-well microbe plates. The method consists in making subsequent dilutions to estimate the density of microorganisms in a liquid medium (Table 3.1), without any direct counting (Wendt-Potthoff and Koschorreck 2002). Samples for MPN analyses were incubated six weeks except those for detecting denitrifying metabolism, which were cultivated for four weeks. Both incubation and the later analysis were conducted in the Helmholtz Environmental Research Centre-UFZ (Department of Lake Research, Magdeburg, Germany). MPNs were calculated with the computer program described by Klee (1993).

The determination of colony-forming units consists in subsequent dilutions of water samples spread onto a variety of overlay media (Table 3.2) designed to promote the

growth of different physiological groups of acidophilic *Bacteria* and *Archaea* (Johnson and Hallberg 2007). The overlay technique (Fig. 3.1) consists in a lower layer inoculated with a heterotroph *Acidocella aromatic* strain PFBC (YE3o, YE4o, SRBo and ABS/TEo) or *Acidiphillium cryptum* strain SJH (iFeo, FeSo, FeTo) and an upper layer with no inoculum (Johnson and Hallberg 2007). These heterotrophs use the compounds released by the agarose hydrolysis at low pH, which inhibit the growth of acidophiles (Küsel 2003, Johnson and Hallberg 2009). The determination of colony-forming units was accomplished in the *Bangor Acidophile Research Team* facilities in Bangor University (Wales, UK). The composition of the overlay media is detailed in the Table 3.2. Plates were incubated aerobically at 30°C for up to 4 weeks; those used to detect phototrophs were cultivated under continuous illumination. Plates to detect anaerobic heterotrophs were placed in a sealed 2.5 L jar under anaerobic conditions created using AnaeroGen® sachets (Oxoid). Pure cultures were obtained by subculturing onto fresh solid media and transferred into corresponding liquid media (Johnson and Hallberg 2007) (Table 3.2).

Table 3.1. Media used for the MPN technique.

Target microbial metabolisms	pH media	Reference
Media for growing anaerobic bacteria		
Acidophilic Fe ³⁺ reduction	2.3	Küsel et al. 1999
Neutrophilic Fe ³⁺ reduction	6.0	Wendt-Potthoff et al. 2012
Neutrophilic sulphate reduction	6.0	Meier et al. 2004
Acid tolerant sulphate reduction	3.0	Meier et al. 2004
Denitrification	7.0	Saitoh et al. 2003
Mixotrophic nitrate reducers Fe and 2 ⁺ oxidation	4.0	Hauck et al. 2001
Lithotrophic nitrate reducers and Fe ²⁺ oxidation	4.0	Hauck et al. 2001
Media for growing aerobic bacteria		
Acidophilic Fe ²⁺ oxidation	1.8	Wendt-Potthoff et al. 2012
Acidophilic thiosulphate oxidation	4.5	Meier et al. 2004
Ammonia oxidation	7.8	Koschorreck and Darwich 2003
Media for growing bacteria under microaerobic conditions		
Fe ²⁺ oxidation	4.0	Hauck et al. 2001 and Benz et al. 1998

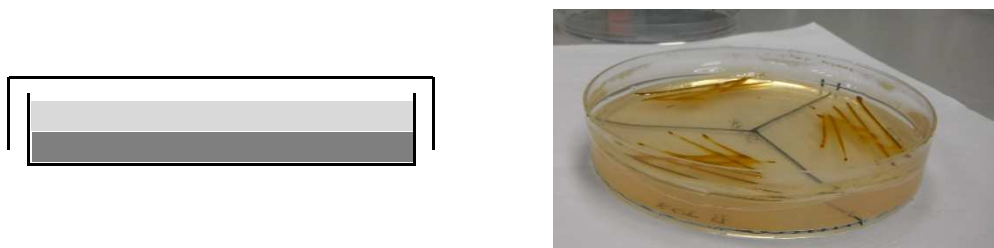


Figure 3.1. Petri dish for overlay technique scheme (left) and photograph (right) showing iron-oxidizing bacteria colonies growing on the overlay surface (FeSo). Dark grey, bottom layer inoculated with *Acidocella aromatic* strain PFBC (YE3o, YE4o, SRBo and ABS/TEo) or *Acidiphillum cryptum* strain SJH (iFeo, FeSo, FeTo). Light grey, cell-free over layer.

Table 3.2. Media used for the direct counting technique and for the sub-cultivation of the isolates.

Solid media	Substrate	pH	Liquid media	pH	Substrate	Targeted metabolism
iFeo	Ferrous iron	2.5	iFe	1.9	Ferrous iron	Iron oxidation
FeSo	Ferrous iron Yeast extract Tetrathionate	2.5	10Fe/YE	2.0	Ferrous iron Yeast extract	Sulphur/Iron oxidation
FeTo	Ferrous iron Thiosulphate	4.5	FeThio	3.5	Ferrous iron Thiosulphate	pH>4.0 iron/sulphur oxidation
YE3o/YE4o	Yeast extract	3.0/4.0	YE	3.5/4.0	Yeast extract	Heterotrophy
SRBo	Sulphate Yeast extract Glycerol	3.5	SRB	3.5	Sulphate Yeast extract Glycerol	Sulphate reduction/anaerobic heterotrophy
ABS/TEo		2.5	ABS+/TE	2.8		Photosynthesis

3.2.4 BIOMOLECULAR ANALYSES

Biomolecular analyses were carried out at Bangor University. DNA was extracted from the membrane filters or sediments using MoBio “ultraclean soil DNA isolation kits”, following the manufacturer’s instruction. Ribosomal RNA genes (eukaryotic 18S rRNA gene and 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 and the 1387R, for 16S rRNA archaeal DNA the 20F and the 915R (Table 3.3 and Table 3.4). For eukaryotes, the primers used were EukF and EukR described by Rowe et al. (2007) and Kay et al. (2013). Ribosomal RNA genes amplification was carried out in triplicate to

minimise PCR bias using Cy5-labelled forward primer for terminal restriction enzyme fragment length polymorphism (T-RFLP) analyses. PCR products were purified using SureClean reagent (Bioline Reagents Ltd., UK) according to the manufacturer's instructions and re-suspended in 20 μ L sterile MilliQ water (Kay et al. 2014).

Table 3.3. PCR primer used for different genes amplification.

Primer	Sequence (5' - 3')	Reference
EukF	ACCTGGTTGATCCTGCCAG	Mariner et al. 2008
EukR	TGATCCTTCYGCAGGTTAC	Mariner et al. 2008
Arch 20F	TCCGGTTGATCCYGCCRG	Kay et al. 2013
Arch 915R	GTGCTCCCCCGCCAATTCCT	Kay et al. 2013
27F	AGAGTTTGATCMTGGCTCAG	Lane 1991
1387R	GGGCGGWGTGTACAAGGC	Marchesi et al. 1998
DSR1 F deg BU	CAYTGGAARCAYGGYGG	Modified after Klein et al. 2001
DSR4 R deg	GTGTARCAGTTDCCRCA	Klein et al. 2001
APS7-FBU	GGGMYTGTCCGCYATYAA	Friedrich 2002 (K.B. Hallberg, personal communication)
APS8-RBU	GCWCATRTCGARGAAGTCTTC	Friedrich 2002 (K.B. Hallberg, personal communication)
mcrAF	GGTGGTGMTMGATTACACARTAYGCWACAGC	Luton et al. 2002
mcrAR	TTCATTGCRTAGTTWGGRTAGTT	Luton et al. 2002

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 (Hallberg et al. 2006).

Specific genes to detect sulphate reduction and methanogenesis were amplified on the DNA extracted from membrane filters by PCR (Table 3.4). These genes were: the

dissimilatory sulphite reductase genes (*dsrAB* gene), that catalyse the reduction of sulphite to sulphide during the anaerobic reduction of sulphate (Klein et al. 2001); the adenosine-5'-phosphosulphate (APS) reductase gene (*apsA* gene), key enzyme present in all sulphate-respiring prokaryotes (Friedrich 2002); the methyl coenzyme-M reductase gene (*mcrA* gene), a common enzyme in methanogens, which catalyses the reduction of a methyl group bound to coenzyme-M with the release of methane (Luton et al. 2002). The primers used were (Table 3.3): DSR1 F deg BU (forward) and DSR4 R deg (reverse) for the *dsrAB* gene; APS7-F (forward) and APS8-R (reverse) for the *apsA* gene; and *mcrAF* (forward) and *mcrAR* (reverse) for the *mcrA* gene.

Table 3.4. Polymerase chain reaction (PCR) procedures for the different analysed genes.

Gene	PCR settings
Bacterial 16S rRNA	95°C (5 min), 30 cycles at 95°C (30 s), 55°C (30 s) and 72°C (1.5 min). Final extension at 72°C (10 min)
Archaeal 16S rRNA	95°C (5 min), 30 cycles at 95°C (30 s), 62°C (30 s) and 72°C (1.0 min). Final extension at 72°C (10 min)
18S rRNA	95°C (5 min), 35 cycles at 95°C (30 s), 59°C (30 s) and 72°C (2.0 min). Final extension at 72°C (10 min)
<i>dsrAB</i>	95°C (5 min), 35 cycles at 95°C (30 s), 54°C (30 s) and 72°C (2.0 min). Final extension at 72°C (10 min)
<i>apsA</i>	95°C (5 min), 35 cycles at 95°C (30 s), 45°C (30 s) and 72°C (1.0 min). Final extension at 72°C (10 min)
<i>mcrA</i>	95°C (5 min), 5 cycles at 95°C (30 s), 55°C (30 s, at 0.1%) and 72°C (30 s). 36 cycles at 95°C (30 s), 55°C (30 s, at 0.1%) and 72°C (30 s). Final extension at 72°C (10 min)

Bacterial isolates lysates were prepared from single colonies or from harvested biomass from liquid cultures with SDS (0.25% sodium dodecyl sulphate in 0.5 M NaOH) for 15 minutes at 95°C. For sequencing purposes, the 16S rRNA genes of the isolates were amplified by PCR and using the unlabelled 27F and 1387R primers. PCR products were then sent to Macrogen, Inc. (Korea) to be sequenced.

A clone library was constructed to identify T-RFs, which were not in the databank maintained at Bangor University (Hallberg et al. 2006). Two water samples were selected for *Bacteria* (DNA from 10.5 m depth in Cueva de la Mora, sampled on March and September 2012) and two additional water samples for *Archaea* (DNA from 15 and 35

m depths in Cueva de la Mora, sampled on March 2012). Clone libraries of amplified 16S rRNA gene archaeal genes from sediment samples at 8 and 35 m depths in Cueva de la Mora were also constructed. PCR amplification of both bacterial and archaeal 16S rRNA genes was performed with 27F and 1387R primers for bacterial and 20F and 915R for Archaea (Table 3.3). 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., Kore), (Kay et al. 2013).

Resulting bacterial isolates and clone (bacterial and archaeal) 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.

3.3 RESULTS

3.3.1 WATER MICROBIOLOGY

3.3.1.1 PHOTOTROPHIC COMMUNITY

Concentrations of chlorophyll-a peaked several meters below the lake water surface and immediately above the chemocline in Cueva de la Mora (Fig. 3.2), and just below the maximum concentration of dissolved oxygen, at a depth where the PAR was <2% of that measured close to the lake surface (Fig. 3.3). The chlorophyll-a peaks in the Guadiana pit lake were found, as with Cueva de la Mora, several metres below the water surface (Fig. 3.2), at depths where the PAR was <2% of that immediately below the water surface (Fig. 3.3) and below the maximum dissolved oxygen concentration (Fig. 3.3). Concentrations of chlorophyll-a declined markedly within the chemocline zone and were very small in water below 12 m depth, probably deriving from cells sinking from the photic zone. Chlorophyll-a variations have been reported previously in the Cueva de la Mora pit lake (Sánchez-España et al. 2009) as in the San Telmo pit lake (Fig. 3.2; Sánchez-España personal communication) which is a meromictic pit lake in the IPB (Sánchez-España et al. 2007a). It has been observed that the chlorophyll-a concentration varies

seasonally, during the winter months the peaks are not as evident as during the warmer months, and, sometimes, this chlorophyll-a maximum is inexistent (Fig. 3.2).

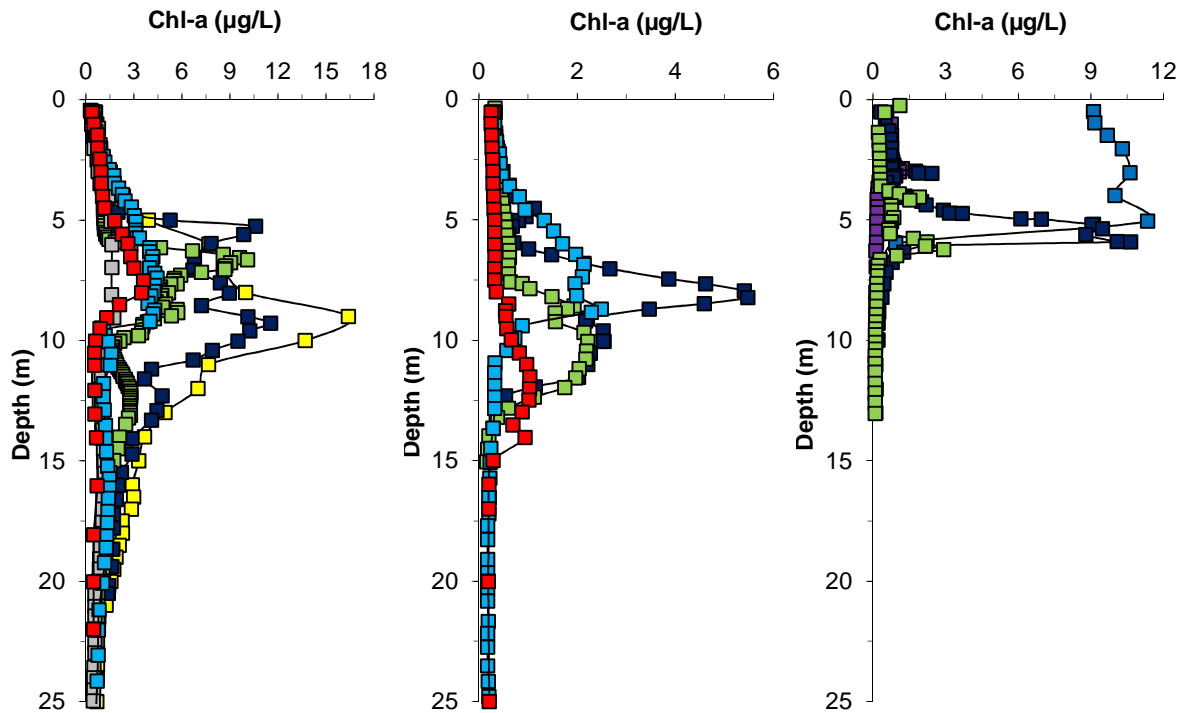


Figure 3.2. Chlorophyll-a in Cueva de la Mora (left) and in Guadiana (middle) and San Telmo (right; Sánchez-España personal communication) pit lakes. ■, November 2010; ■, March 2011; ■, April 2011; ■, July 2011; ■, September 2011; ■, March 2012; ■, September 2012. Note in the graph that only the first 25 m of the lake water columns are represented to distinguish the chlorophyll-a distribution.

Pigment analyses (Fig. 3.4) showed that the main pigment present in the photic zone was chlorophyll-a. However, in Cueva de la Mora chlorophyll-a peak zone, chlorophyll-b was also detected and carotenoids and bacteriochlorophyll-a might also be potential contributors to the pigment bulk at that depth. Pheophytin-a was detected in the Cueva de la Mora sample (12.3 m), which corresponds with the secondary chlorophyll-a maximum present at the same depth (Fig. 3.2). The accumulation of senescent photosynthetic cells in depth would potentially be contributing to this secondary peak (Fig. 3.2).

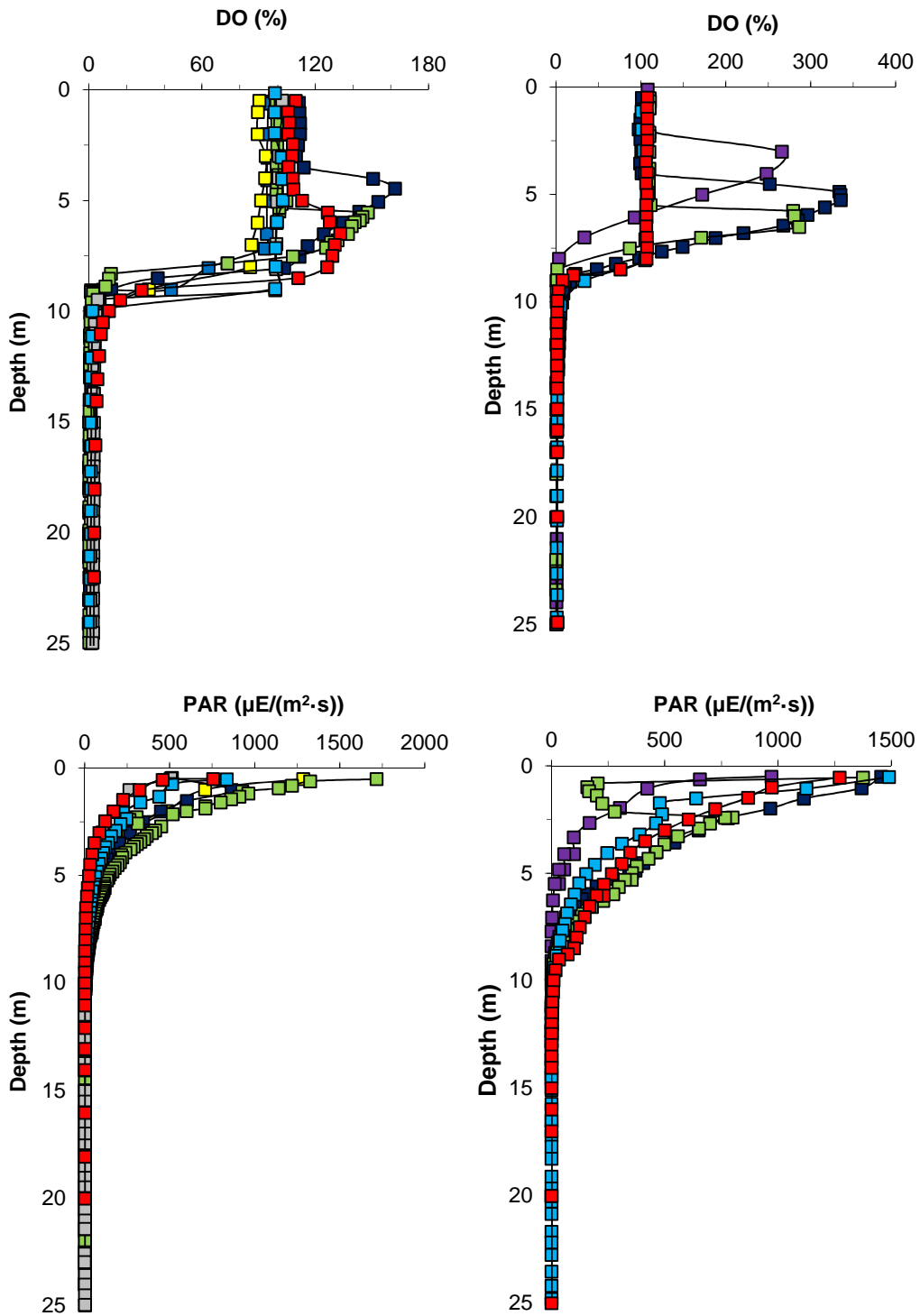


Figure 3.3. Vertical profiles of dissolved oxygen (DO) and photosynthetically-active radiation (PAR) intensity in Cueva de la Mora (left) and in Guadiana (right) pit lakes showing the different layers within these two lakes. ■, November 2010; ■, March 2011; ■, April 2011; ■, July 2011; ■, September 2011; ■, March 2012; ■, September 2012. Note in the graph that only the first 25 m of the lake water columns are represented as both parameters DO and PAR do not change from 10 m downwards.

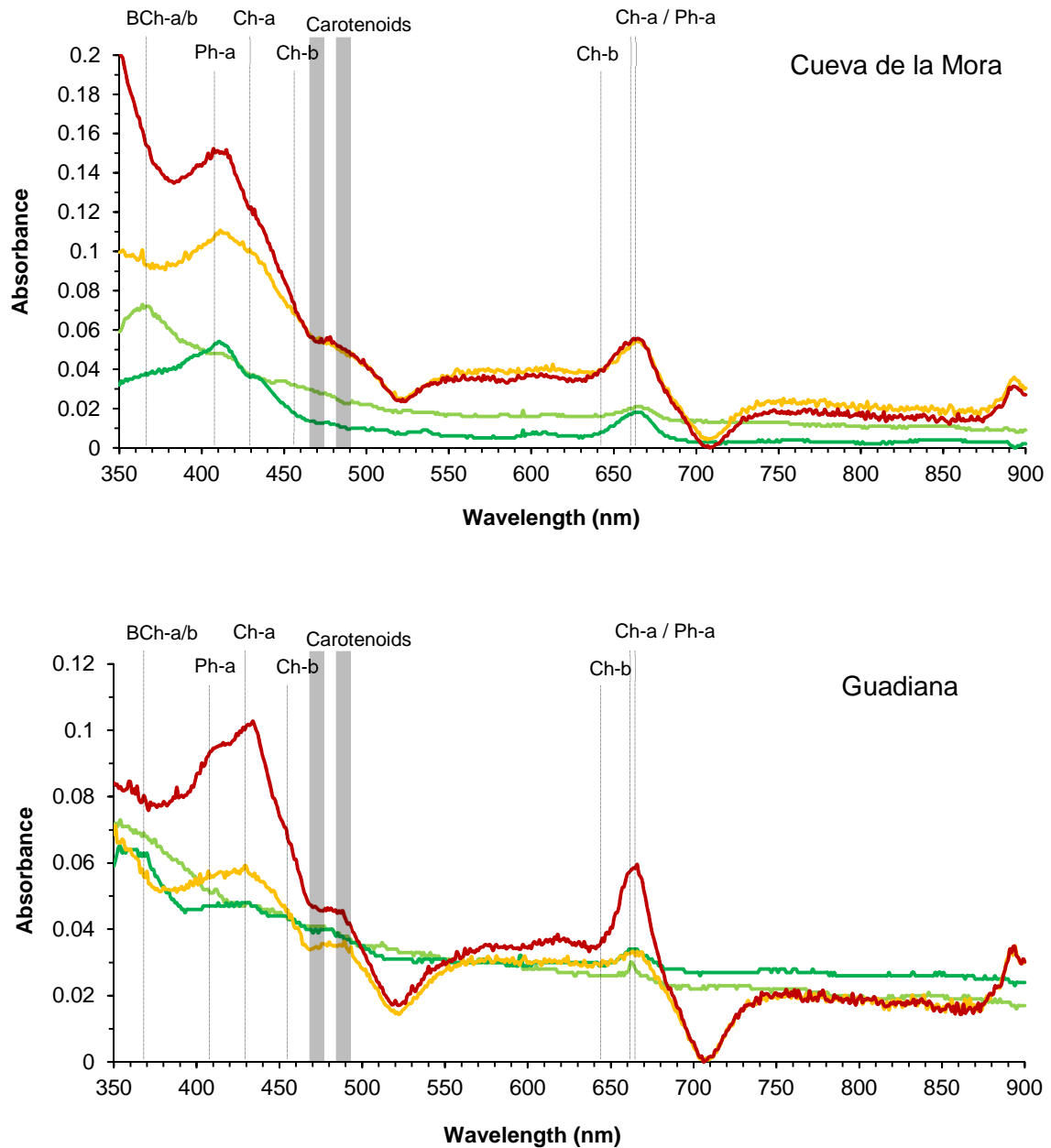


Figure 3.4. Pigment analyses of water samples taken from depths corresponding to the chlorophyll-a profiles major peaks in Cueva de la Mora (CM; top) and in Guadiana (G; bottom) pit lakes on different months. Light green line, July 5.25 m (CM) and 5.3 (G); dark green line, July 12.3 m (CM) and 10 m (G); orange line, September 6.7 m (CM) and 6 m (G); red line line, september 12.3 m (CM) and 10 m (G). Peaks for different pigments extracted with acetone; chlorophyll-a (Ch-a), at 430 and 662 nm; chlorophyll-a (Ch-b), at 456 and 645 nm; pheophytin-a (Ph-a), at 409 and 665 nm; carotenoids, maximum absorption at 450-460 nm and at 480-490 nm and bacteriochlorophyll- a and -b (BCh-a/b) main peak at 368 nm.

Two acidophilic phototrophic microorganisms, *Pseudococcomyxa* sp. (strain AC1; accession number KC155323) and *Auxenochlorella protothecoides* var. *acidicola* (strain CFR26, accession number KM462820) were isolated on solid media from the Cueva de la Mora mixolimnion sampled in March 2012 (isolate AC1) and September 2012 (isolate CFR26). The dominant T-RF found in profiles from the epilimnion of the Guadiana pit lake (Fig. 3.5) was from chloroplast DNA, and corresponded to a micro-algal isolate (*Coccomyxa* sp. AH4; accession number KC155324) that was identical to *Coccomyxa* sp. AC1 isolated from the Cueva de la Mora pit lake. Although the major peaks could not be identified the T-RFLP profiles show that the epilimnetic waters of Guadiana present similar eukaryotic community in both samples (March 2012 and September 2012), whilst the Cueva de la Mora mixolimnion shows variations between the two samples (Fig. 3.5). It is likely that the eukaryotic community presents seasonal variations just like chlorophyll-a profiles show variations in the different samplings (Fig. 3.2).

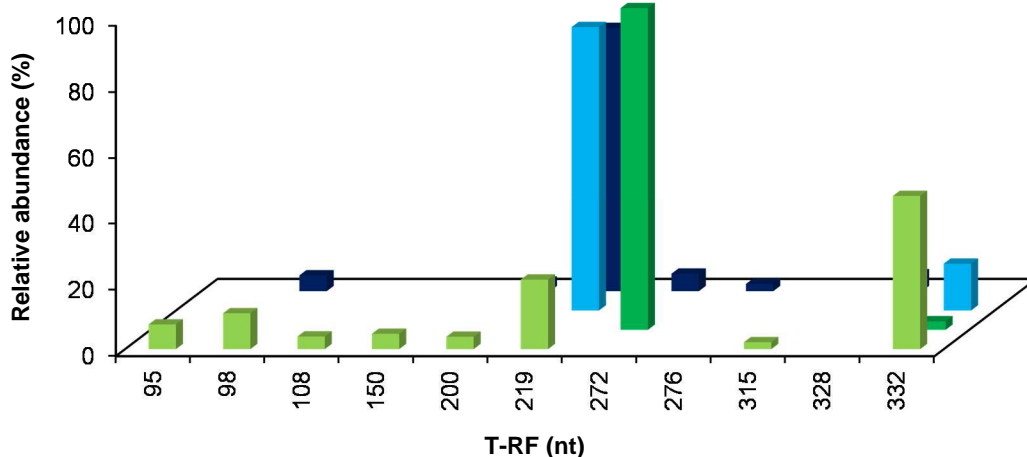


Figure 3.5. T-RFLP profiles of the amplified 18S rRNA gene T (digested with HaeIII) of filtrates from the mixolimnion sampled in March 2012 (light green bars) and September 2012 (dark green bars) of the Cueva de la Mora pit lake and the epilimnion sampled in March 2012 (light blue bars) and September 2012 (blue bars) of the Guadiana pit lake. The major peaks identified corresponded to the isolates AC1, AH1 and CFR26 (220 ± 2 nt). The identity of the 272 ± 2 nt and 332 ± 2 nt peaks is unknown. nt, nucleotide.

Other unidentified phototrophic species were also found in the lake waters as well as other non-phototrophic eukaryotes as ciliates or heliozoans (Fig. 3.6 and Fig. 3.7). One of these phototrophs was similar to *Chlamydomonas* sp., which has been previously

observed in a water sample of the Cueva de la Mora pit lake (Sánchez-España et al. 2009).

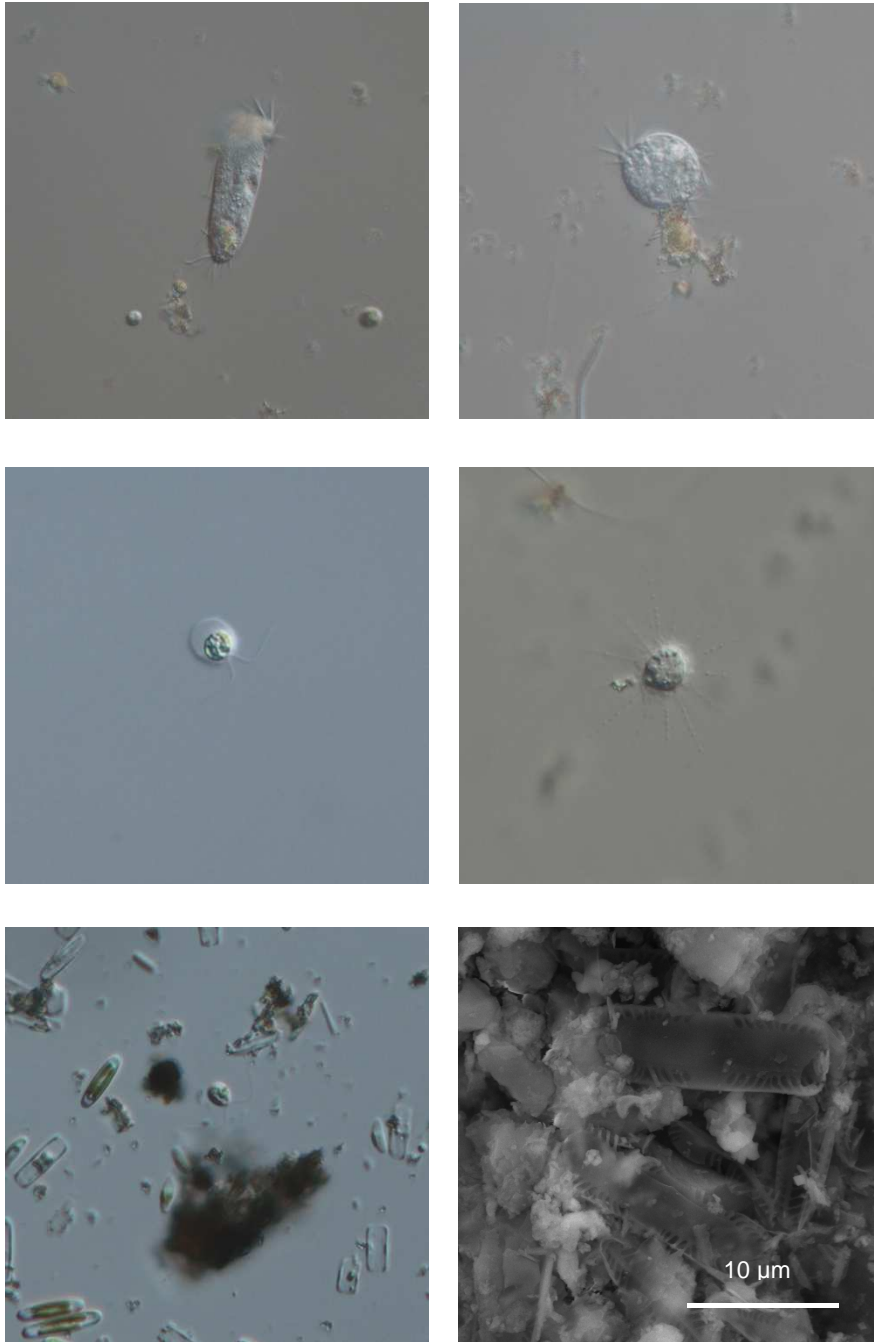


Figure 3.6. Plankton present in water samples (mixolimnion) of the Cueva de la Mora pit lake observed under optical microscope at different magnifications (100-200x) and by scanning electron microscopy (bottom right). Top, Ciliates; middle left, flagellated *Chlamydomonas*-like; middle-right, heliozoan; bottom, diatoms.

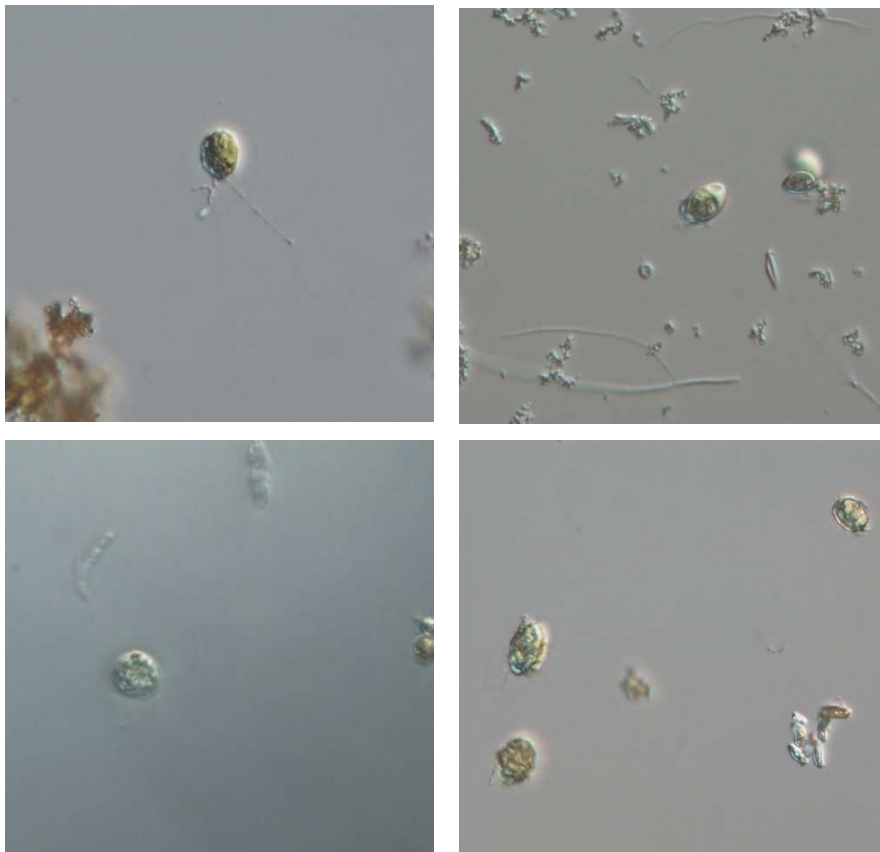


Figure 3.7. Plankton present in water samples (mixolimnion) of the Guadiana pit lake observed under optical microscope at different magnifications (100-200x). Left top, flagellated; right top, planktonic phototroph *Pseudococcomyxa*-like; left bottom, flagellated *Chlamydomonas*-like; right bottom, phototroph.

3.3.1.2 PROKARYOTIC COMPOSITION

Cell counting by the most probable number method shows that both lakes are populated by diverse microbial metabolisms typical of acidic environments (Fig. 3.8). The chemocline of Cueva de la Mora and the hypolimnion of Guadiana are the most inhabited layers where different metabolisms are present, including iron reduction, iron oxidation, sulphur oxidation, and sulphate reduction. Although the *dsrAB* gene (specific gene present in sulphate-reducers) could not be amplified from water samples, the *apsA* gene typical of sulphate-respiring prokaryotes was detected in the Cueva de la Mora chemocline. The *mcrA* gene, which is very commonly gene found in methanogens, was detected in a deep basin sediment sample in the same lake. No acidophilic iron reduction and sulphate-reduction were detected in the monimolimnion zones.

The different bacterial metabolisms are well correlated with the substrates availability, as it will be discussed below. Whereas the upper layer is the most favourable horizon to support the growth of phototrophic microorganisms, the transition layer would be the most favourable environment for the non-phototrophic bacterial population as both oxic and anoxic conditions coexist (Fig. 3.3), and therefore oxidized and reduced compounds are available for bacterial metabolisms (Chapter 2).

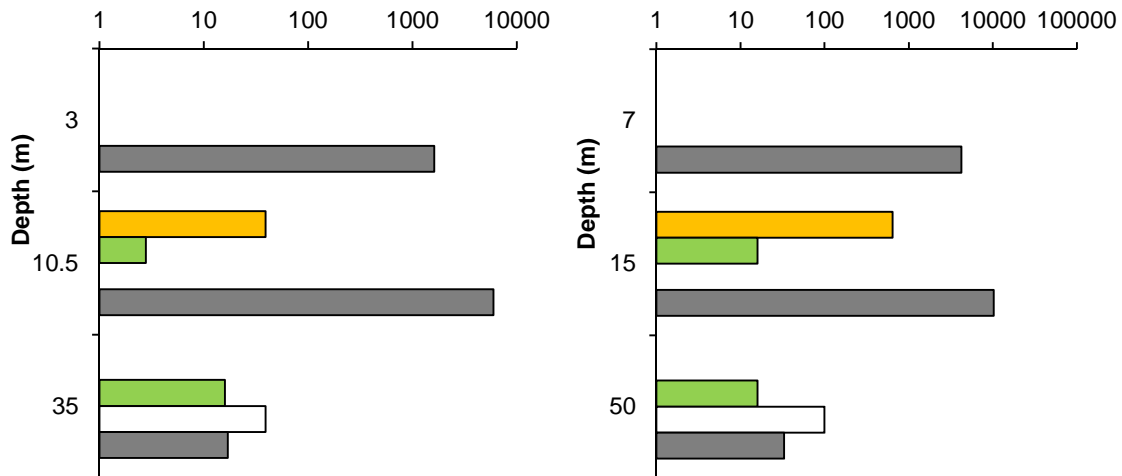


Figure 3.8. Most probable numbers in the Cueva de la Mora (left) and in Guadiana (right) pit lakes in March 2012. Yellow bars, iron oxidation; white bars, sulphur oxidation; green bars, iron reduction; grey bars, sulphate reduction.

3.3.1.2.1 CUEVA DE LA MORA

Numbers of acidophilic iron-oxidizing and heterotrophic bacteria (colony forming units; CFUs) were always greater within the chemocline zone than at other depths in Cueva de la Mora (Fig. 3.9). Aerobic heterotrophic bacteria (obligate aerobic and facultative anaerobe) were invariably the most abundant physiological group in all water samples analysed and were the only prokaryotes isolated from the monimolimnion. Interestingly, anaerobic acidophilic heterotrophs were only isolated from the chemocline zone in this pit lake.

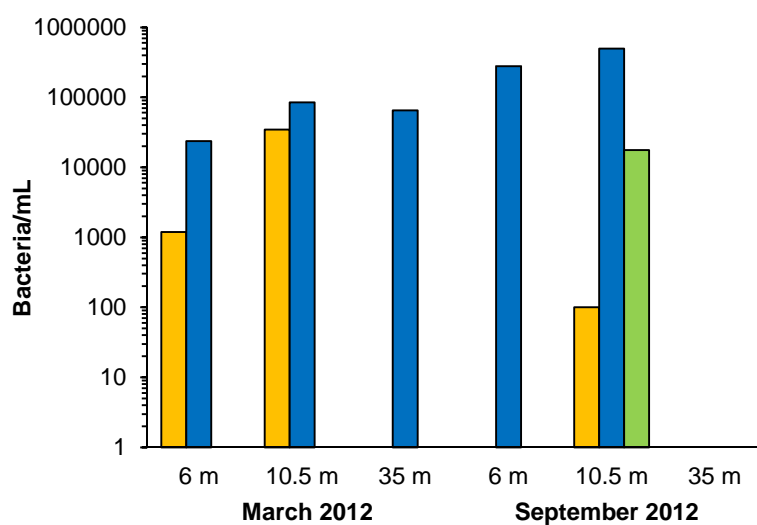


Figure 3.9. Numbers (colony forming units) of acidophilic iron-oxidizing bacteria (yellow bars), aerobic heterotrophic bacteria (blue bars) and anaerobic heterotrophic bacteria (green bars) in waters sampled at different depths within the Cueva de la Mora pit lake during March and September, 2012. No enumeration of anaerobes was carried out with March 2012 samples.

Bacterial biodiversity in the mixolimnion and chemocline zones in Cueva de la Mora, as assessed by T-RFLP analysis, is shown in Fig 3.10. Many of T-RFs correlated either with entries in a database maintained at Bangor University or with acidophilic isolates obtained from the lake, implying that acidophiles accounted for most of the indigenous microbial communities in this pit lake. However, the dominant T-RFs from both the March and September 2012 chemocline samples corresponded to bacterial clones that were not isolated (clone 4-20_mar12 and clone 3_sept12 respectively). The T-RF/clone detected in the March 2012 (clone 4-20_mar12) sample had 80% similarity of its 16S rRNA gene to *Thermosporothrix hazakensis* (Yabe et al. 2010) and could not be affiliated to any known lineage of the domain Bacteria (Fig. 3.11; Table A.3.1 in appendix). The new division into which this clone was placed ("TM7"; Hugenholtz et al. 2001) currently contains no cultivated species, and the role of the bacterium represented by this clone in catalysing dissimilatory redox transformations of iron or sulphur is unknown. A different dominant T-RF was obtained from amplified DNA from the Cueva de la Mora chemocline sampled in September 2012. The 16S rRNA gene sequence of the corresponding clone was 95% similar to that of *Desulfomonile tiedjei* (Fig. 3.11; Table A.3.1 in the appendix). Clones related to this genus of sulphate-reducing bacteria have also been obtained from

other acidic environments (Loy et al. 2004, Koschorreck et al. 2010, Sánchez-Andrea et al. 2012).

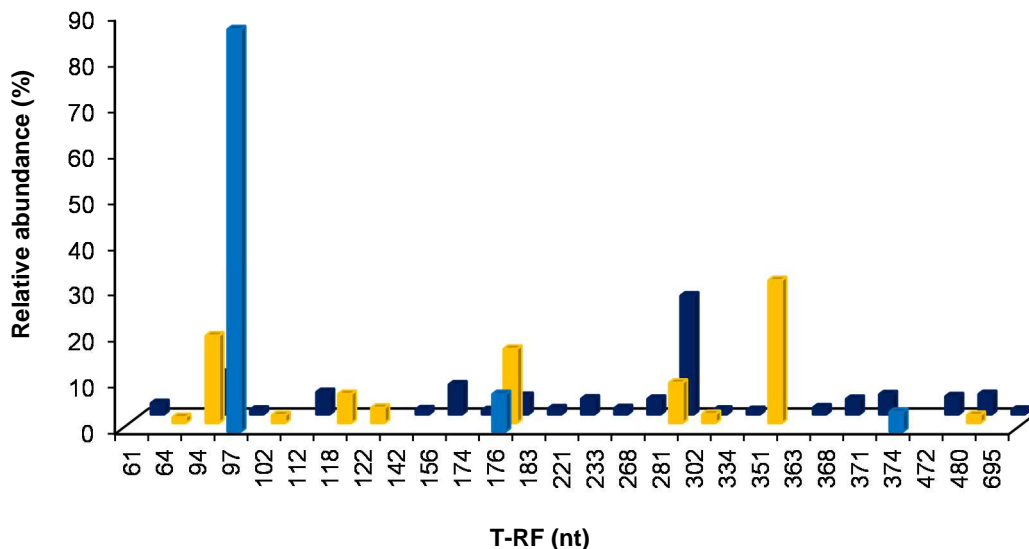
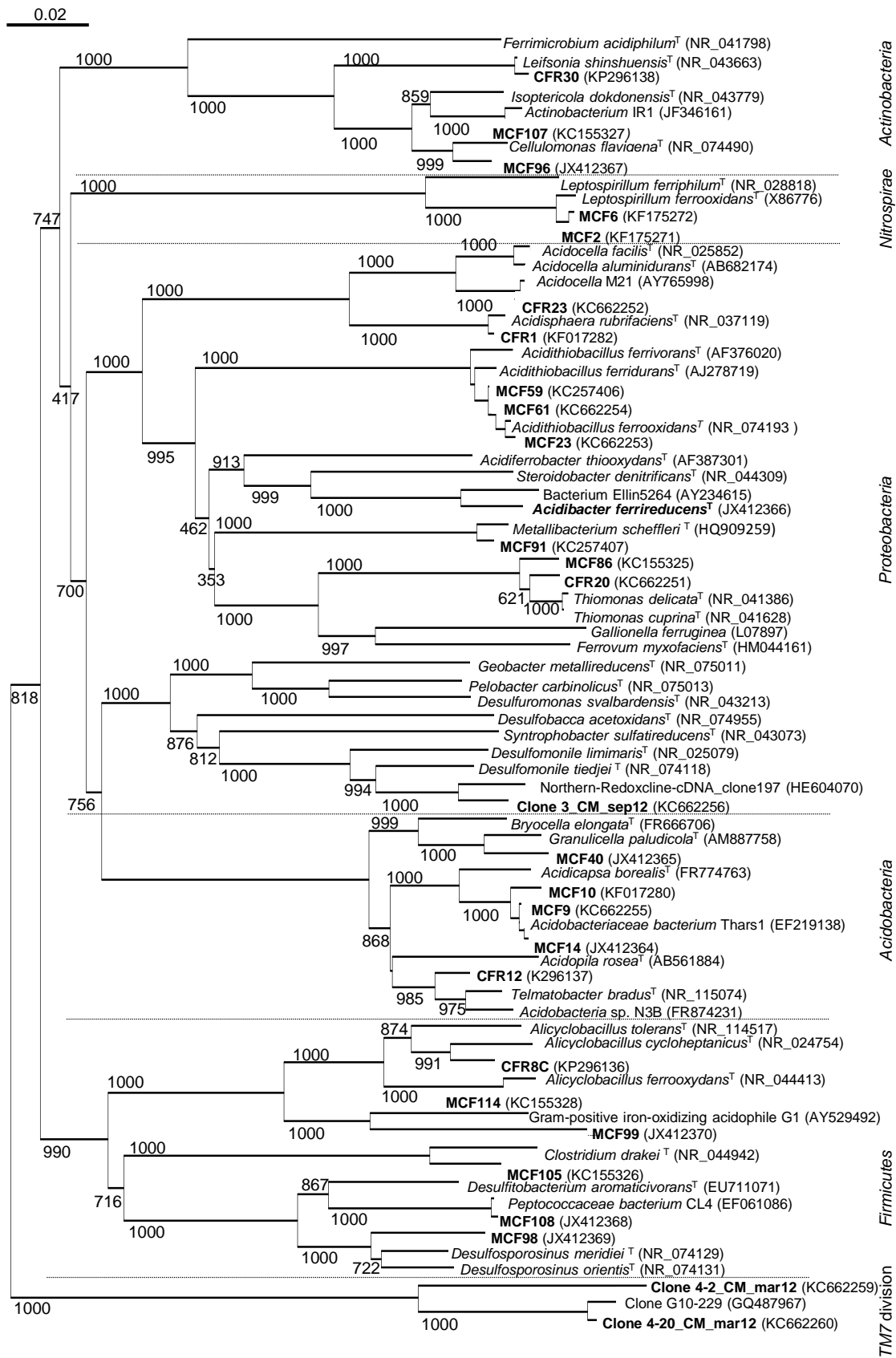


Figure 3.10. T-RFLP profiles of amplified bacterial 16S rRNA gene (digested with CfoI) of filtrates from the mixolimnion (yellow bars) and chemocline (dark blue bars) of the Cueva de la Mora pit lake sampled in March 2012, and in the chemocline (light blue bars) sampled in September 2012. The major peaks identified corresponded to clone 3_sep12 (97 ± 2 nt), chloroplast 16S rRNA (351 ± 2 nt), clone 4-20_mar12 (282 ± 2 nt), *Acidisphaera rubrifaciens* (177 ± 2 nt), isolates MCF9 and MCF10 (93 ± 2 nt).

Archaeal 16S rRNA genes were amplified only from water samples in the monimolimnion zone in Cueva de la Mora sampled in March 2012. The T-RFLP profiles obtained were far less complex than the bacterial profiles, with only one peak being found in HaeIII digests and 3-5 peaks with AluI-digested DNA products. Two different clones were identified in a library generated from amplified archaeal genes from this zone (clone 6A-1 and clone 5A-1, Fig. 3.12; Table A.3.2 in the appendix). Both belong to the *Euryarchaeota* phylum, but neither could be ascribed to any known archaeal species. Similar clones have previously been identified in mud samples from an acidic (pH 2.5, 28°C) spring in Japan (Kato et al. 2011). As was the case with bacteria, no archaeal 16S rRNA genes could be amplified from monimolimnetic water sampled in September 2012.



Next page
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Figure 3.11. Phylogenetic tree showing the relationship between bacterial isolates and clones (in bold) obtained from the Cueva de la Mora and Guadiana pit lakes, to known species. The tree was rooted with the 16S rRNA gene sequence from *Metallosphaera sedula* (GenBank accession number U3836).

GenBank accession numbers are given in parentheses.

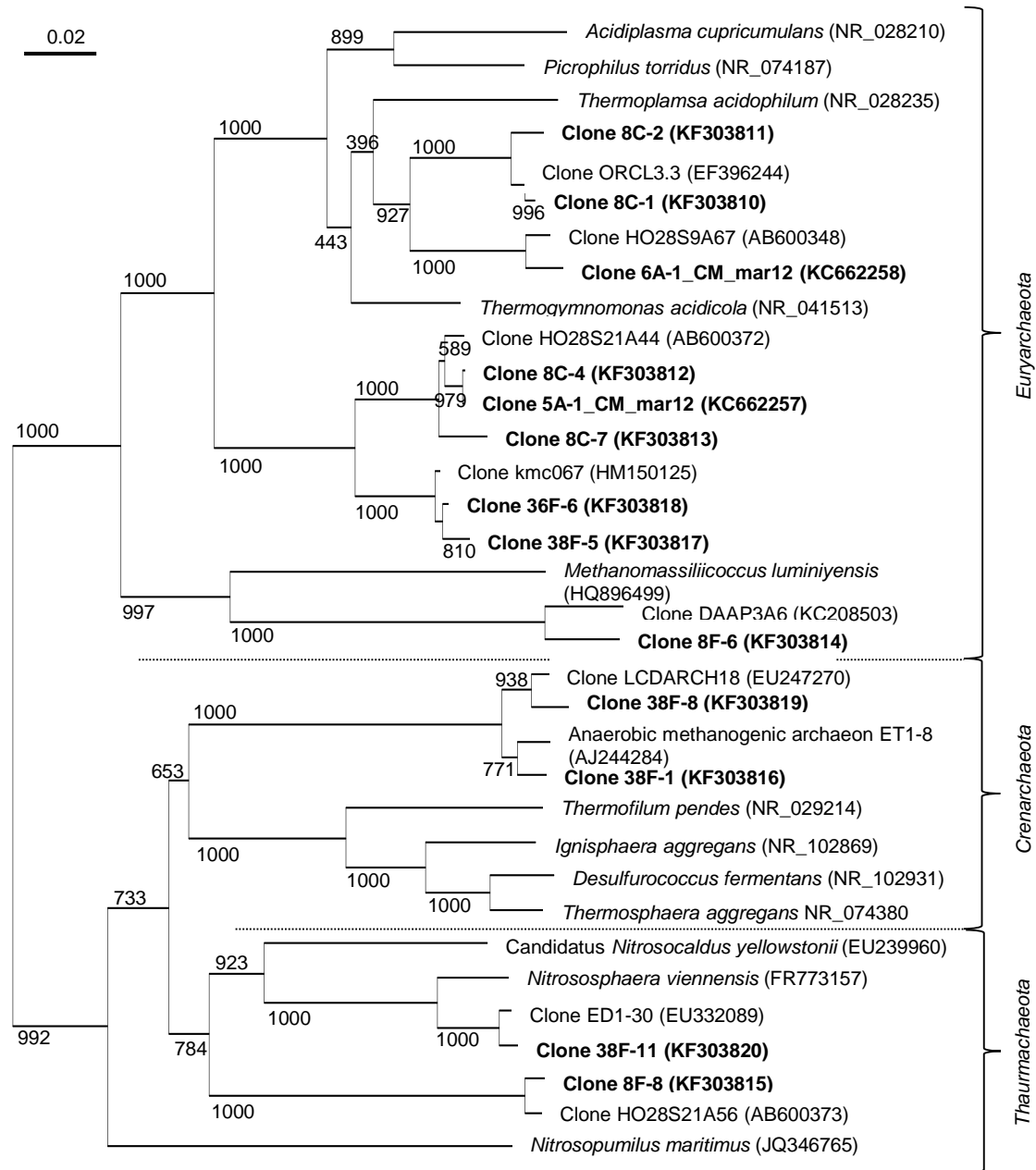


Figure 3.12. Phylogenetic tree showing the relationship between archaeal clones (in bold) obtained from the water column (clones 6A-1_CM_Mar12 and 5A-1_CM_mar12) and shelf-level (clones 8F-) and basin-level (clones 38F-) sediment samples, to known species. The tree was rooted with the 16S rRNA gene sequence from *Leptospirillum ferrooxidans* (GenBank accession number X86776). GenBank accession numbers are given in parentheses.

3.3.1.2.2 GUADIANA PIT LAKE

The dominant bacteria found in the T-RFLP profiles were identified as acidophilic heterotrophs: *Acidobacteriaceae* and *Acidocella* in the March 2012 sample, and *Acidobacteriaceae* and *Acidisphaera* in the September 2012 sample (Fig. 3.13).

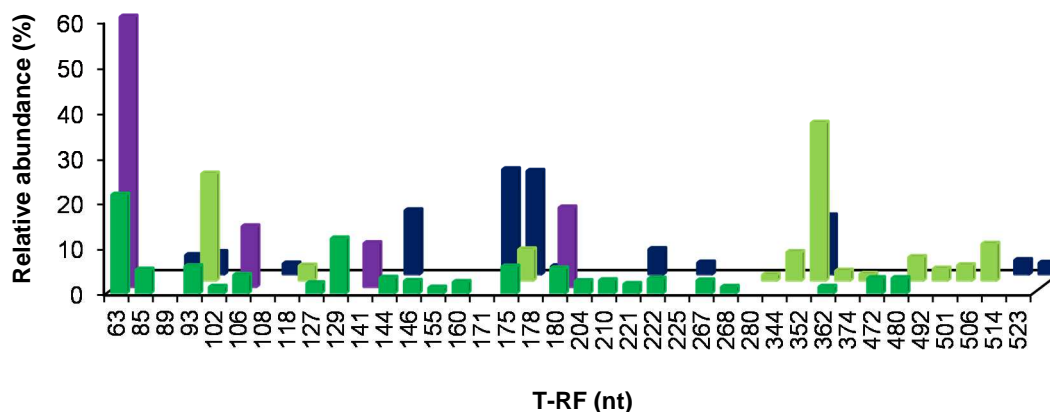


Figure 3.13. T-RFLP profiles of amplified bacterial 16S rRNA gene (digested with CfoI) of filtrates water from the epilimnion (light green bars) and the hypolimnion (dark green bars) of the Guadiana pit lake sampled in March 2012, and the epilimnion (blue bars) and the hypolimnion (purple bars) sampled in September 2012. The major peaks identified corresponded to *Metallibacterium scheffleri* (63 ± 2 nt), chloroplast 16S rRNA (351 ± 2 nt), isolates MCF14 and MCF40 (93 ± 2 nt), *Acidisphaera rubrifaciens* (177 ± 2 nt), MCF86 (144 ± 2 nt).

The hypolimnion was the most microbially-diverse layer in the Guadiana pit lake. Total numbers of acidophilic bacteria isolated from this zone were also greater than at other depths in March 2012 samples, though were slightly less than those in the epilimnion in the September samples (Fig. 3.14). On both sampling occasions the dominant bacteria identified in T-RFLP profiles from the hypolimnion corresponded to isolates that were very closely related to *Metallibacterium scheffleri* (strain MCF91), and *Thiomonas* sp. (strain MCF86; see Table A.3.1 in the appendix). A number of other acidophilic bacteria known, or demonstrated in this study, to be actively involved in catalysing biogeochemical transformations of iron and sulphur were also isolated from the hypolimnion of Guadiana as described below. Several bacterial isolates were obtained from the epilimnion and hypolimnion of this lake (Fig. 3.11 and Table A.3.1 in the appendix), whereas no DNA was amplified or isolates obtained from the

monimolimnion layer of Guadiana on either sampling occasion. In addition, no archaeal genes were amplified from any water sample from the Guadiana pit lake on either sampling occasion.

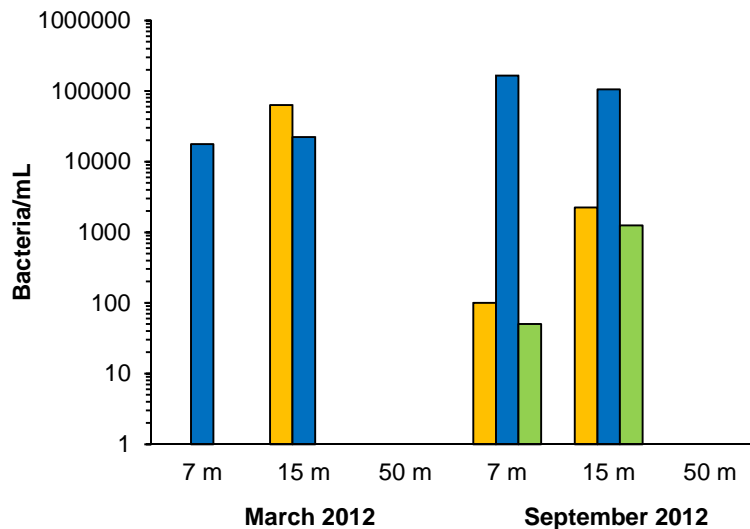


Figure 3.14. Numbers (colony forming units) of acidophilic iron-oxidizing bacteria (yellow bars), aerobic heterotrophic bacteria (blue bars) and anaerobic heterotrophic bacteria (green bars) in waters sampled at different depths within the Guadiana pit lake during March and September 2012. No enumeration of anaerobes was carried out with March 2012 samples.

3.3.2 SEDIMENT MICROBIOLOGY: CUEVA DE LA MORA

The bacterial T-RFLP profiles from the shore sediment samples at Cueva de la Mora were dominated by two heterotrophs, *Acidicapsa* sp., and *Metallibacterium scheffleri*, and the obligately aerobic iron-oxidizer *Leptospirillum ferrooxidans* (Fig. 3.15). In contrast, a *Desulfosporosinus* sp. as well as actinobacteria were found in the basin sediment sample, although many T-RFs were not identified (Fig. 3.16).

Although no archaea was isolated from the Cueva de la Mora sediments, as elsewhere, archaeal 16S rRNA genes were successfully amplified from both shore-level and deep basin-level samples. Clone libraries generated using amplified genes identified six distinct clones from the shelf-level sediment, and a further five from the basin-level sediment (Fig. 3.12; see also Table A.3.2 in the appendix). Although seven of the clones were euryarchaeotes (like those in the water column, though one of the sediment clones

was most closely related to a methanogenic archaeon), two were crenarchaeotes and the other two representative of the recently-described phylum *Thaumarchaeota* (Fig. 3.12).

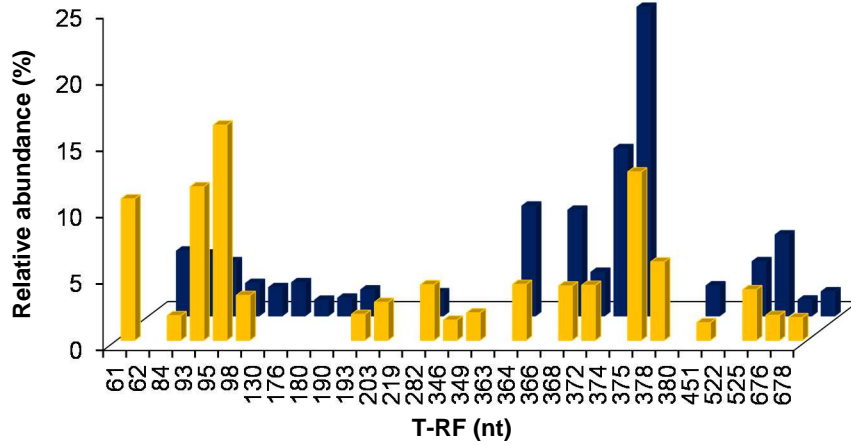


Figure 3.15. T-RFLP profiles of bacterial 16S rRNA gene (digested with CfoI) of interstitial water extracted from 4-5 cm (orange bars) and 7-9 cm (blue bars) layer depths in a shore sediment sample (8 m) in the Cueva de la Mora pit lake. The major peaks identified corresponded to the isolates MCF9 and MCF10 (93 ± 2 nt), *Leptospirillum ferrooxidans* (374 ± 2 nt), *Alicyclobacillus ferrooxydans* and *Metallibacterium scheffleri* (61 ± 2 nt), *Ferrithrix thermotolerans* (366 ± 2 nt), MCF105 (226 ± 2 nt), *Desulfosporosinus acididurans* (596 ± 2 nt).

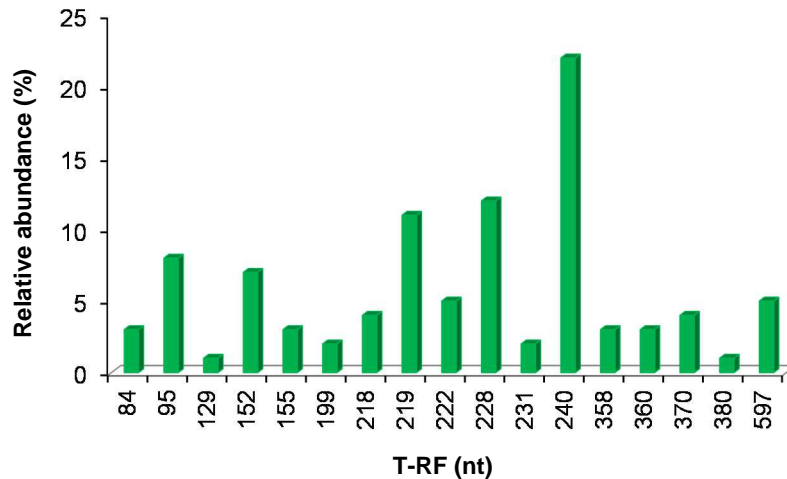


Figure 3.16. T-RFLP profiles of bacterial 16S rRNA gene (digested with CfoI) of interstitial water extracted from 8-10 cm from a deep basin sediment sample (38 m) in the Cueva de la Mora pit lake. The major peaks identified corresponded to the isolates MCF9 and MCF10 (93 ± 2 nt), MCF105 (226 ± 2 nt), MCF108 (358 ± 2 nt), and *Desulfosporosinus acididurans* (596 ± 2 nt), The identity of the 240 nt peak is not known.

3.3.3 IDENTIFICATION AND CHARACTERISTICS OF ACIDOPHILIC BACTERIA ISOLATED FROM THE CUEVA DE LA MORA AND GUADIANA PIT LAKES

A large number (>100) of bacteria were isolated from lake water from the two pit lakes, and from sediment samples from Cueva de la Mora. Those identified as distinct strains or species, and whose 16S rRNA genes were successfully amplified and sequenced, are listed in Table A.3.1 of the appendix. While many of these could be affiliated to known species of acidophilic bacteria, some of them were identified as novel species (Fig. 3.11, see also Table A.3.1 of the appendix). These included *Acidibacter* (*Ab.*) *ferrireducens* (MCF85), a representative of a novel genus of γ -*Proteobacteria* which is being characterized (Falagán and Johnson 2014) and *Firmicute* MCF99, whose closest relative (93% gene identity) is an unclassified *Alicyclobacillus* sp. (BGR73; Breuker et al. 2009).

Many of the bacterial isolates were either known (from previous studies), or demonstrated in the current study, to catalyse dissimilatory redox transformations of iron and/or sulphur in acidic environments (Table 3.5). These included ferrous iron-oxidizers (*Leptospirillum ferrooxidans*, *Acidithiobacillus* (*At.*) *ferrooxidans*, *At. ferrivorans* and *Alicyclobacillus* (*Al.*) *ferrooxydans*), ferric iron-reducers (*Acidobacteriaceae* isolates MCF9, MCF10 and MCF14; *Metallibacterium scheffleri*, *Acidiphilium* sp., *Acidocella* sp., *Ab. ferrireducens*, *At. ferrooxidans* and *At. ferrivorans*), sulphur-oxidizers (*Thiomonas* sp., *At. ferrooxidans* and *At. ferrivorans*) and sulphate-reducers (*Desulfosporosinus* and “*Desulfobacillus*” spp.).

3.4 DISCUSSION

The Cueva de la Mora and Guadiana pit lakes were chosen among many others in the IPB because they display the most dramatic vertical gradients of pH and water chemistry of all pit lakes studied to date. They thus represent world-class examples of meromictic pit lake stratification with complex (sub-layered) internal structure of their anoxic water bodies. The stratification patterns found in the two pit lakes studied demonstrates the co-existence of three ‘environments’ with highly contrasting physicochemical characteristics within the same water body.

Table 3.5. Physiological characteristics of acidophilic bacteria found in the Cueva de la Mora and Guadiana pit lakes (Johnson and Hallberg 2009). OA, obligate aerobe; OA(M), aerobic/micro-aerobic; FAn, facultative anaerobe; OAn, obligate anaerobe; Het, obligate heterotroph; Auto, obligate autotroph; Fac, facultative autotroph/heterotroph; Fe³⁺-red, ferric iron reducer; Fe²⁺-ox, ferrous iron oxidizer; S_{red-ox}, sulphur-oxidizer; SO₄²⁻-red, sulphate reducer.

		O ₂ -requirement	C-source	Fe/S transformations
Cueva de la Mora				
Mixolimnion	<i>Acidobacteriaceae</i>	OA(M)	Het	Fe ³⁺ -red*
	<i>Ac. rubrifaciens</i>	OA	Het	
Chemocline	<i>At. ferrooxidans</i>	FAn	Auto	Fe ²⁺ -ox Fe ³⁺ -red S _{red-ox}
	<i>L. ferrooxidans</i>	OA	Auto	Fe ²⁺ -ox
	<i>At. ferrivorans</i>	FAn	Auto	Fe ²⁺ -ox Fe ³⁺ -red S _{red-ox}
	<i>Thiomonas</i> sp.	OA	Fac	S _{red-ox}
	<i>Desulfomonile</i> sp	OAn	Het	SO ₄ ²⁻ -red
	<i>Acidocella</i> sp.	FAn	Het	Fe ³⁺ -red
	<i>Acidiphilium</i> sp.	FAn	Het	Fe ³⁺ -red
Monimolimnion	Unknown organisms			
Guadiana				
Epilimnion	<i>Acidobacteriaceae</i>	OA(M)	Het	Fe ³⁺ -red
	<i>Ac. rubrifaciens</i>	OA	Het	
	<i>At. ferrooxidans</i>	FAn	Auto	Fe ²⁺ -ox Fe ³⁺ -red S _{red-ox}
	<i>Acidocella</i> sp.	FAn	Het	Fe ³⁺ -red
	<i>Thiomonas</i> sp.	OA	Fac	S _{red-ox}
Hypolimnion	<i>Mt. sheffleri</i>	FAn	Het	Fe ³⁺ -red
	<i>At. ferrooxidans</i>	FAn	Auto	Fe ²⁺ -ox Fe ³⁺ -red S _{red-ox}
	<i>L. ferrooxidans</i>	OA	Auto	Fe ²⁺ -ox
	<i>At. ferrivorans</i>	FAn	Auto	Fe ²⁺ -ox Fe ³⁺ -red S _{red-ox}
	<i>Thiomonas</i> sp.	OA	Fac	S _{red-ox}
	<i>Acidobacteriaceae</i>	OA(M)	Het	Fe ³⁺ -red
	Gammaproteobacteria MCF85	FAn	Het	Fe ³⁺ -red
	<i>Granulicella</i> sp.	OA	Het	
Monimolimnion	No data			

Table 3.5. Continued.

		O ₂ -requirement	C-source	Fe/S transformations
Cueva de la Mora				
Shelf shore sediments	<i>Firmicute</i> spp.	OAn		
	<i>Al. ferrooxydans</i>	OA	Het	Fe ²⁺ -ox S _{red} -OX
	<i>Clostridium</i> sp.	OAn	Het	
Deep basin sediments	<i>Clostridium</i> sp.	OAn	Het	
	<i>Desulfosporosinus</i> sp.	OAn	Het	SO ₄ ²⁻ -red
	<i>Cellulomonas</i> sp.	Fan	Het	
	" <i>Desulfobacillus</i> " spp.	OAn	Het	SO ₄ ²⁻ -red
	<i>Al. tolerans</i>	OA	Het	Fe ²⁺ -ox Fe ³⁺ -red S _{red} -OX

The three layers of the lakes (mixolimnion, chemocline and monimolimnion in Cueva de la Mora, and epilimnion, hypolimnion and monimolimnion in Guadiana) are interconnected by a coupled vertical mass of transfer, including downwards settling of metal oxyhydroxide precipitates and upwards flux of solutes driven by ionic diffusion (Chapter 2). While thermal stratification of lakes is a well-known phenomenon, the presence of large concentrations of two elements (iron and sulphur), which can be both oxidized and reduced by many species of prokaryotes indigenous to moderately and extremely acidic environments gives added complexity to the structure of pit lakes formed in abandoned opencast metal and coal mine sites.

A major apparent anomaly in both pit lakes examined was that the greatest accumulation of algal biomass was found at depths where PAR was <2% of that close to the water surface (primary peak found above the chemocline). The secondary chlorophyll-a peak found deeper at 12 ± 0.5 m might be caused by accumulation of senescent cells (phototrophic microorganisms) in this layer and the subsequent degradation of pigments such as phaeophytin-a. Besides needing solar energy, acidophilic micro-algae also require carbon dioxide and inorganic nutrients. While dissolved carbon dioxide is far more abundant below the chemocline than in the surface of Cueva de la Mora (55-60 mg/L CO₂ in upper 5 m compared to 300 mg/L CO₂ at 10.5 m and >1,300 mg/L CO₂ at 35 m depth; Sánchez-España et al. 2013), CO₂ concentrations in the surface layers would be sufficient to sustain algal growth.

Concentrations of inorganic nitrogen were similar throughout the water column in this lake, and differences in bio-available nitrogen were thought unlikely to be the reason why algae accumulated ~8 m below the water surface. In contrast, concentrations of phosphate, a macro-nutrient that is often poorly bio-available in oxidized, acidic, iron-rich waters due to ferric phosphate being highly insoluble ($K_{sp} = 1.3 \times 10^{-22}$) were two orders of magnitude less in the chemocline layer than in the monimolimnion in Cueva de la Mora, and even lower in the mixolimnion. This phenomenon appears to be due to the diffusion of soluble phosphate from the monimolimnion, the microbial reduction of soluble and solid phase ferric iron (including, presumably, ferric phosphate) in the chemocline zone (as evidenced by the occurrence of both ionic forms of this metal as well as by the presence of many different species of bacteria that can catalyse this reaction) and the predominance of ferric iron in aerated surface waters. This observation corresponds with previous studies pointing to P-limitation for phytoplanktonic growth in acidic pit lakes in Germany (e.g. Kleeberg 2013).

Acidophilic micro-algae generate oxygen and are the major source of organic carbon (e.g. exudates and lysates) supporting the growth of heterotrophic acidophiles in many low pH environments (Ñancuqueo and Johnson 2012a). The most abundant bacteria in the mixolimnion (from both cultivation-based and molecular analyses) were moderately acidophilic heterotrophs (*Acidobacteriaceae*). Although three of the four acidobacteria isolates obtained from the pit lakes could, like other strains, catalyse the dissimilatory reduction of ferric iron under micro-aerobic conditions (Johnson and Hallberg 2009), they would be unlikely to be doing so to a significant extent in this aerobic water layer, as evidenced by the absence of detectable ferrous iron in this zone. Water below the chlorophyll-a peak (e.g. the chemocline) is the interface between the oxygenated mixolimnion and anoxic monimolimnion, and was populated by obligate aerobes (e.g. *Leptospirillum ferrooxidans*), facultative anaerobes (e.g. *At. ferrooxidans*) and obligate anaerobes (e.g. *Desulfomonile* sp.) suggesting the existence of micro-environments of varying oxygen contents within this zone. The chemocline also contains both oxidized and reduced iron (ferric and ferrous) and sulphur (sulphate and hydrogen sulphide; Wendt-Potthoff et al. 2012, Diez-Ercilla et al. 2014) in contrast to both the oxidized mixolimnion and the reduced monimolimnion, indicating that this transitional zone is where redox transformations of these two elements are at their most dynamic. This study has identified bacteria within the Cueva de la Mora chemocline that can mediate all of the transformations corresponding to the MPN experiment results obtained. These include *At. ferrooxidans*, which can both oxidize and reduce iron, as well as oxidize

reduced sulphur, and others that have more specialized roles in iron/sulphur biogeochemistry (e.g. ferrous iron-oxidizing *Leptospirillum*, sulphur-oxidizing *Thiomonas*, and sulphate-reducing *Desulfomonile*). Many different species of bacteria found in this zone (e.g. *Acidocella* sp., *Acidobacteriaceae* and *Acidithiobacillus* sp.) can reduce ferric iron under micro-aerobic and anaerobic conditions. Heterotrophic acidophiles were, as in the mixolimnion, found to be more abundant than chemolithoautotrophs in the chemocline. This suggests that organic carbon compounds (derived from acidophilic micro-algae, which are the main primary producers in this pit lake) rather than inorganic electron donors (ferrous iron and reduced sulphur) are the dominant electron donors used by bacteria in Cueva de la Mora, in contrast to some other metal-rich acidic environments that have been described (e.g. Bond et al. 2000, Kay et al. 2013).

The only terminal electron acceptor (other than carbon dioxide) present in significant concentrations in the anoxic monimolimnion zone in Cueva de la Mora, is sulphate, and it might therefore be anticipated that sulphate-reducing bacteria (SRB) would proliferate in this water layer. Although there are evidences of SRB in the lower monimolimnion (Wendt-Potthoff et al. 2012), no detection by cultivation-based nor cultivation-independent methods have shown the presence of SRB in the monimolimnion, even though species that can grow in acidic, metal-rich waters elsewhere in the IPB have been previously isolated and characterized (Dopson and Johnson 2012). In contrast, the presence of SRB in the chemocline was determined using cultivation-independent methods. The reason for this apparent anomaly is likely to be that suitable electron donors are not present in sufficient concentrations in the monimolimnion. SRB tend to use a limited range of small molecular weight organic substrates and hydrogen as energy sources, and it is likely that the former, originating principally from phototrophic primary producers, are metabolized by the relatively large population of heterotrophic acidophiles occupying the chemocline zone leaving a very small amount of organic substrates available for the inhabiting bacteria of the monimolimnion, resulting in a very low cell density layer. A schematic representation of the iron, sulphur and carbon cycles in the Cueva de la Mora pit lake, and of the bacteria identified as mediating the various biogeochemical transformations identified, is shown in Fig. 3.17.

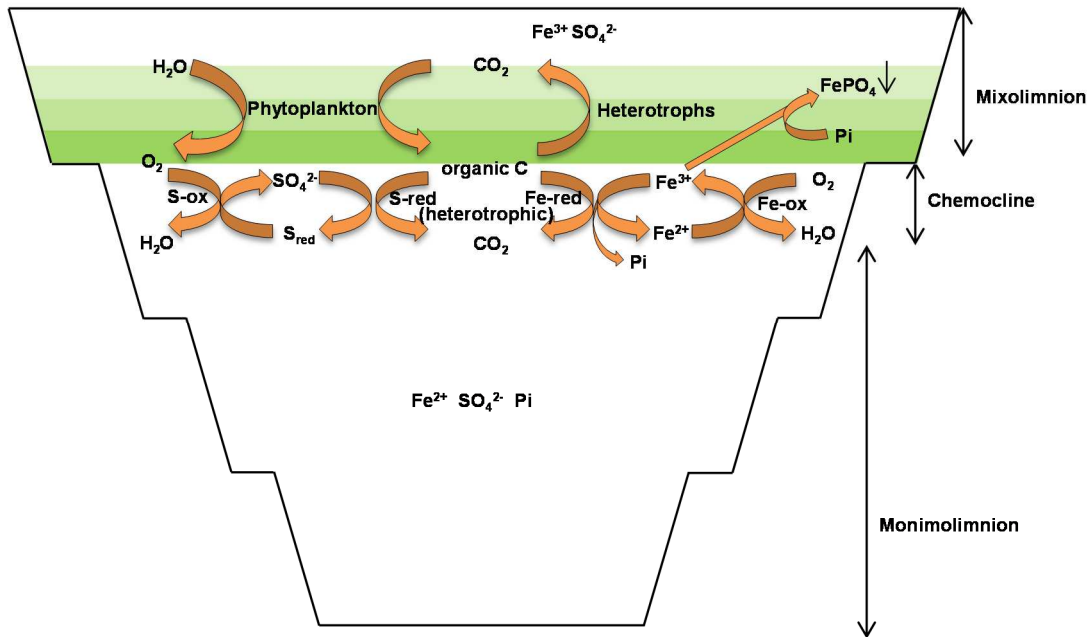


Figure 3.17. Schematic representation of the transformations of carbon, iron and sulphur in the stratified Cueva de la Mora pit lake, and of the bacteria identified as mediating these processes. Pi, inorganic phosphorus; -red, reduction; -ox, oxidation.

The Guadiana pit lake presents a similar, though in some ways contrasting, scenario. This is a younger lake that is still filling with rainfall and groundwater. The hypolimnion in Guadiana is analogous to the chemocline zone in Cueva de la Mora in that it is located between an aerobic (epilimnion) and anoxic (monimolimnion) zone, though it is mostly homogeneous (and therefore classed as a mixolimnion layer) with a sharp transition between aerobic and anaerobic water close to its surface. The hypolimnion is also the zone that displays the greatest diversity of indigenous microorganisms and, on one of the sampling occasions, the largest number of cultivatable bacteria. In March, chlorophyll-a levels peaked just above the hypolimnion, again analogous to the situation at Cueva de la Mora, though in September most algal biomass was found within the upper part of this zone. Again, it was thought that phosphate availability dictated where maximum algal growth occurred within this lake, though concentrations of this macro-nutrient were lower than in Cueva de la Mora throughout the water column. Micro-algae would have had the same impact on indirectly promoting the redox transformations of iron and sulphur via their generation of oxygen and organic carbon as described for the Cueva de la Mora pit lake. Interestingly, even though SRB were detected by the MPN technique no SRB were detected in, or isolated from, the

Guadiana pit lake in any other occasion. A similar situation occurs in the monimolimnion; although there are evidences of sulphur-related and iron metabolisms (Table 3.5), no isolates were obtained from this layer suggesting that the low cell density makes the isolation of the microorganisms very difficult. These findings are in good agreement with the lack of H₂S and/or metal sulphides observed in the water column of this pit lake (Sanchez-España et al. 2014b) as opposed to Cueva de la Mora, where both H₂S and metal sulphides precipitates are abundant in the chemocline and upper monimolimnion (Diez-Ercilla et al. 2014). Overall, the results obtained suggest that the lower phosphate content in the epilimnion and/or the scarcity of suitable organic carbon found in the monimolimnion of the Guadiana pit lake could be strongly determining the growth of SRB populations.

While all of the prokaryotes that were isolated from both pit lakes were bacteria, archaeal genes were detected in both the water column and sediments in Cueva de la Mora. Although all of the archaeal clones were too distantly related to known species for any firm conclusions to be drawn about their physiological activities, some broad inferences can be made. Both clones obtained from the water column, and also most of those from the sediment samples, were euryarchaeotes, distantly related to acidophilic archaea (*Thermoplasma* and *Thermogymnomonas* spp.), though, interestingly, one was more closely related to a methanogen (*Methanomassiliicoccus luminiyensis*; Table A.3.2 in the appendix). No meaningful affiliation could be made with the two crenarchaeotal clones, though acidophilic crenarchaeotes tend to be thermo-acidophiles (e.g. *Sulfolobus* and *Acidianus* spp.). Most intriguing was the finding that two of the sediment clones grouped within the *Thaumarchaeota*. All of the validated species in this recently-described phylum are chemolitho-autotrophic ammonium-oxidizers (Pester et al. 2011), and it is reasonable to speculate that the clones identified represent archaea that are mediating the transformation of nitrogen in the Cueva de la Mora sediments. Many diverse and apparently novel species of archaea have been detected in extremely acidic environments (e.g. Justice et al. 2012), though relatively few have been isolated from non-thermal low pH sites. Their presence in Cueva de la Mora suggests that the unknown archaea are acidophiles and new approaches will be required to isolate them from this pit lake and similar environments.

The bacterial population of the deep basin sediments of the Cueva de la Mora pit lake is slightly different than that found in the shore sediments. Whilst the latest is populated by iron oxidizers, aerobic heterotrophs and some sulphate reducers, the deep

basin sediments where inhabited exclusively by anaerobes (Table A.3.1 in the appendix). In addition, the different archaeal clones obtained, although distantly related to other known archaeal species as above mentioned, suggest the presence of other microbial metabolisms, which contribute to the sediments chemistry, as methanogenesis and possibly ammonium-oxidizers. Methane has been detected in the monimolimnetic waters of the Cueva de la Mora pit lake (Wendt-Potthoff et al. 2012), as well as ammonium (Wendt-Potthoff et al. 2012, Sánchez-España et al. 2013, Chapter 2), which suggests the presence of methanogens and ammonium-oxidizers in the pit lake sediments. These findings provide evidence that the microbial composition in sediments of Cueva de la Mora is complex, and more studies are required to understand better the microbial activity in sediments of acidic mine pit lakes.

The biogeochemistry and microbiology of two other pit lakes (Nuestra Señora del Carmen and Concepción) in the IPB have been recently reported by Santofimia et al. (2013). These lakes differed from those described in the present study in that they were less markedly stratified and contained far smaller concentrations of dissolved metals. The macro-nutrients nitrogen and phosphorus were more uniformly distributed throughout the water column of the latter pit lakes, so that the critical role of phosphorus, found in the present study at Cueva de la Mora, was not apparent. Indigenous microorganisms were identified from clone libraries, and no isolates were obtained and characterized. Models to explain the biogeochemical cycling of iron and sulphur in the pit lakes studied were presented by Santofimia et al. (2013) which show some similarity and some differences to that proposed in the present study. In the four lakes, the chlorophyll-a maximum was found close or by the transition layer, this corresponds with the assumption that the nutrients availability is conditioning the development of the phototrophic community as it was previously proposed. The Nuestra Señora del Carmen pit lake water column chemistry is dominated by the iron cycle although some sulphur cycling takes place at the lake bottom; the authors suggest that the predominance of iron oxidation or iron reduction within the water column would depend on the seasonal mixing. Guadiana and mainly Cueva de la Mora are more metabolically diverse lakes than Concepción and Nuestra Señora del Carmen as both iron and sulphur cycles are well established in the water column (Table 3.6), mainly in the chemocline (Cueva de la Mora) and hypolimnion (Guadiana).

Table 3.6. Microbial metabolisms in the IPB lakes. *, chemocline in Cueva de la Mora, and in Concepción and hypolimnion in Guadiana, Nuestra Señora del Carmen present a very narrow and shallow chemocline, Lake 111 does not present stratification. Fe ox, iron oxidation; Fe red, iron reduction; Het, heterotrophy; PS, photosynthesis; S ox/red, sulphur oxidation and sulphur reduction; SO₄²⁻ red, sulphate reduction; S ox, sulphur oxidation.

	Cueva de la Mora	Guadiana	Nuestra Señora del Carmen ^a	Concepción ^a	Lake 111 ^b
Mixolimnion	Fe ox Fe red Het PS	Fe red Het PS	Fe ox Het PS	Het PS	
Transition layer*	Fe ox Fe red S ox/red SO ₄ ²⁻ red Het	Fe ox Fe red S ox/red Het	n.d.	Fe ox Fe red Het	Fe ox Fe red S ox/red Het
Monimolimnion	un.	un.	Fe ox Fe red S ox/red	Fe ox Fe red S ox ⁺	

un., undetermined.

n.d., not data.

^a, Santofimia et al. 2013.

^b, Wendt-Potthoff 2013.

Bacterial diversity in the Concepción pit lake was mainly dominated by heterotrophic microorganisms, and the microbial composition of this lake is fairly conditioned by the runoff water and the groundwater. Although the chemocline is the most bacterially diverse layer within the lake (Table 3.7) as in the cases of Cueva de la Mora or Guadiana (Santofimia et al. 2013). The Nuestra Señora del Carmen anoxic monimolimnion shows more diversity probably caused by the diverse energy sources (Santofimia et al. 2013). Compared to German pit lakes, that do not show a permanent water column stratification, the bacterial diversity is, in general, similar to the Spanish acidic mine pit lakes. Within the water column of the Lake 111 (Lusatia, Germany), the typical acidophiles, as *Acidithiobacillus*, *Leptospirillum*, *Acidiphilium* or *Acidobacteria* among others, were detected, in addition to other genera that are not typical from acidic environments such as *Legionella* or *Nitrosomonas* (Wendt-Potthoff 2013). Surprisingly the Berkley pit lake (Butte, USA) does not seem to be bacterial diverse as the above mentioned acidic pit lakes, and the acidophile, *Acidithiobacillus ferrooxidans* has been the only isolate obtained from the lake waters. The unsuccessful isolation of other bacteria might be due to the ambient conditions, which could not be favourable for bacterial growth, and to date,

no other bacterial isolates have been obtained even from the lake sediments (Gammons and Tucci 2013).

Table 3.7. Bacterial taxonomic groups identified in four metal mine pit lakes in the Iberian Pyrite Belt (Cueva de la Mora, CM; Guadiana, G; Nuestra Señora del Carmen, NSC; Concepción, C) and one coal mine pit lake in the Lusatia mining district in Germany (ML111).

	CM	G	NSC ^a	C ^a	ML111 ^b
Depth (m)	38	>60	34	16	12
Estimated volume (m³)	282,300	>268,082	79,500	72,500	
Mixolimnion	<i>α-Proteobacteria</i> <i>Acidobacteria</i>	<i>α-Proteobacteria</i> <i>Acidobacteria</i>	<i>α-Proteobacteria</i> <i>γ-Proteobacteria</i> <i>Nitrospira</i> <i>Planctomycetia</i>	<i>α-Proteobacteria</i> <i>γ-Proteobacteria</i> <i>δ-proteobacteria</i> <i>Firmicutes</i> <i>Actinobacteria</i> Candidate division TM6	<i>α-Proteobacteria</i> <i>β-Proteobacteria</i> <i>γ-Proteobacteria</i> <i>Acidobacteria</i> <i>Actinobacteria</i> <i>Nitrospira</i> <i>Acidithiobacilli</i> <i>Firmicutes</i> <i>γ-timonadetes</i>
Transition layer*	<i>Acidithiobacillia</i> <i>α-Proteobacteria</i> <i>δ-Proteobacteria</i> <i>Nitrospira</i> <i>Acidobacteria</i> Candidate division TM7	<i>Nitrospira</i> <i>Acidithiobacillia</i> <i>γ-Proteobacteria</i> <i>β-roteobacteria</i>	n.d.	<i>α-Proteobacteria</i> <i>γ-Proteobacteria</i> <i>δ-Proteobacteria</i> <i>Acidobacteria</i> <i>Actinobacteria</i> Candidate division TM6	
Monimolimnion	un.	un.	<i>Nitrospira</i> <i>Actinobacteria</i> <i>Chloroflexi</i>	<i>β-Proteobacteria</i> <i>Actinobacteria</i>	

*, chemocline in CM, CN and hypolimnion in G, NSC present a very narrow and shallow chemocline. Lake 111 does not present permanent stratification.

un., undetermined.

n.d. no data.

^a, Santofimia et al. 2013.

^b, Wendt-Potthoff 2013.

3.5 CONCLUSIONS

This study has shown how the availability of phosphorous determines the microbial distribution within the pit lakes' waters. The phytoplankton community is concentrated in the upper 8-10 m of the water column in the both studied meromictic pit lakes. Phytoplanktonic distribution is in turn conditioned by light intensity and nutrients availability, mainly phosphorus, which is bound to ferric iron in oxic layers and free in the anoxic layer. The transition layers, chemocline (Cueva de la Mora) and the hypolimnion (Guadiana), are found to be the most microbially diverse in both lakes. The simultaneous availability of oxidized and reduced forms of substrates in the transition layer and the

import of organic carbon from the upper layers make these transition layers a very favourable environment for many bacterial species.

While the phytoplankton inhabits the upper layer of the Cueva de la Mora and Guadiana pit lakes, the iron and the sulphur biogeochemical cycles drive the redox chemistry of the transition layers, which is evidenced by that iron and sulphur oxidizers as well as iron and sulphate reducers are the dominant metabolisms in this zone.

This study has revealed that the bacterial and archaeal communities in pit lake sediments can be quite complex, and that more research should be done to fully understand the microbial community inhabiting them.

More studies will also be necessary to gain a better knowledge of the microbiology of these extreme environments and fully understand the role that microorganisms play in the water and sediments hydrogeochemistry.

3.6 APPENDIX

Table A.3.1. Identities of bacterial isolates and cloned genes obtained from the Cueva de la Mora and Guadiana pit lakes. C, Cueva de la Mora water column; G, Guadiana water column; C_{sed1}, Cueva de la Mora sediment (8 m); C_{sed2}, Cueva de la Mora sediment (38 m). GenBank accession numbers are shown in parentheses.

Isolate/clone designation	Closest relative	% Identity (16S rRNA gene)	Reference
Bacterial isolates			
MCF2 ^G (KF175272)	<i>Leptospirillum ferrooxidans</i> (X86776)	99	Hippe 2000
MCF6 ^C (KF175272)	<i>Leptospirillum ferrooxidans</i> (X86776)	99	Hippe 2000
MCF9 ^C (KC662255)	MCF10 (KF017280)	99	This study
	MCF14 (JX412364)	99	This study
MCF10 ^C (KF017280)	<i>Acidicapsa borealis</i> (FR774763)	97	Kulichevskaya et al. 2012
	MCF14 (JX412364)	99	This study
MCF14 ^G (JX412364)	<i>Acidicapsa borealis</i> (FR774763)	97	Kulichevskaya et al. 2012
MCF23 ^G (KC662253)	<i>Acidithiobacillus ferrooxidans</i> (NR_074193)	100	Valdés et al. 2008
MCF40 ^G (KF017281)	<i>Granulicella paludicola</i> (AM887758)	97	Pankratov & Dedysh 2010
MCF59 ^G (KC257406)	<i>Acidithiobacillus ferrooxidans</i> strain CF3 (JX442933)	100	Hedrich & Johnson 2013
MCF61 ^G (KC662254)	<i>Acidithiobacillus ferrooxidans</i> strain IESL32 (HQ902071)	100	Galleguillos et al. 2009
MCF85 ^G (JX412366)	<i>Steroidobacter denitrificans</i> (NR_044309)	90	Fahrbach et al. 2008
MCF86 ^G (KC155325)	<i>Thiomonas cuprina</i> (NR_041628)	98	Kelly et al. 2007
	CFR20 (KC662251)	98	This study
MCF91 ^G (KC257407)	<i>Metallibacterium scheffleri</i> (HQ909259)	99	Ziegler et al. 2013
MCF96 ^{Csed2} (JX412367)	<i>Cellulomonas flavigena</i> (NR_074490)	98	Abt et al. 2010
MCF98 ^{Csed1} (JX412369)	<i>Desulfosporosinus meridiei</i> (NR_074129)	96	Robertson et al. 2001
	<i>Desulfosporosinus orientis</i> (NR_074131)	96	Stackebrandt et al. 1997

MCF99 ^{Csed1} (JX412370)	Gram-positive iron-oxidizing acidophile G1 (AY529492)	93	Johnson et al. 2005
	<i>Alicyclobacillus contaminans</i> (NR_041475)	90	Goto et al. 2007
MCF105 ^{Csed2} (KC155326)	<i>Clostridium drakei</i> (NR_044942)	96	Liou et al. 2005
MCF107 ^{Csed2} (KC155327)	Actinobacterium IR1 (JF346161)	99	Ñancucheo & Johnson 2012b Yoon et al. 2006 Abt et al. 2010
	<i>Isoptericola dokdonensis</i> (NR_043779)	97	
	<i>Cellulomonas flavigena</i> (N_074490)	97	
MCF108 ^{Csed2} (JX412368)	Peptococcaceae bacterium CL4 (EF061086)	99	Rowe et al. 2007
	<i>Desulfitobacterium aromaticivorans</i> (EU711071)	94	Kunapuli et al. 2010
MCF114 ^{Csed1} (KC155328)	<i>Alicyclobacillus ferrooxydans</i> (NR_044413)	99	Jiang et al. 2008
CFR1 ^C (KF017282)	<i>Acidisphaera rubrifaciens</i> (NR_037119)	99	Hiraishi et al. 2000
CFR8C ^{Csed1} (KP296136)	<i>Alicyclobacillus cycloheptanicus</i> (NR_024754)	97	Wisotzkey et al. 1992
CFR12 ^{Csed1} (KP296137)	Uncultured Acidobacterium sp. clone JL123_1 (HQ730651)	99	Sánchez-Andrea et al. 2011
	<i>Telmatobacter bradus</i> (NR_115074)	97	Pankratov et al. 2012
CFR17 ^{Csed1} (KF303821)	Uncultured bacterium clone RT6-ant10-e12- S (JF737903)	99	García-Moyano et al. 2012
	<i>Desulfosporosinus burensis</i> (NR_109421)	98	Mayeux et al. 2013
CFR20 ^G (KC552251)	<i>Thiomonas delicata</i> (NR_041386)	98	Katayama et al. 2006
	<i>Thiomonas cuprina</i> (NR_041628)	98	Kelly et al. 2007
CFR23 ^C (KC662252)	<i>Acidocella</i> sp. M21 (AY765998)	100	Hallberg et al. 2006
	<i>Acidocella aluminiidurans</i> (AB362219)	97	Kimoto et al. 2010
	<i>Acidocella facilis</i> (NR_025852)	97	Kishimoto et al. 1995
CFR30 ^C (KP296138)	<i>Leifsonia shinshuensis</i> (NR_043663)	99	Suzuki et al. 1999
Bacterial clones			
Clone 4- 2_CM_mar12 (KC662259) ("TM7 division")	Clone ERF-G10 from <i>Erica andevalensis</i> rhizosphere in Río Tinto (DQ906081)	94	Mirete et al. 2007
	<i>Spirulina laxissima</i> (DQ393278)	77	Choi et al. 2012
Clone 4- 20_CM_mar12 (KC662260)	Uncultured bacterium clone G10-229 16S ribosomal RNA gene (GQ487967)	99	Unpublished
	<i>Thermosporothrix hazakensis</i> (AB500145)	80	Yabe et al. 2010

Clone 3_CM_sep12 (KC662256)	Clone SblDsmon4 from an acidic German fen (AY167450)	99	Loy et al. 2004
	<i>Desulfomonile tiedjei</i> (NR_074118)	95	DeWeerd et al. 1990

Table A.3.2. Identities of cloned archaeal genes obtained from the Cueva de la Mora. C, Cueva de la Mora water column; C_{sed1}, Cueva de la Mora sediment (8 m); C_{sed2}, Cueva de la Mora sediment (38 m). GenBank accession numbers are shown in parentheses.

Isolate/clone designation	Closest relative	% Identity (16S rRNA gene)	Reference
Clone 5A-1_CM_mar12 (KC662257) ^C	Clone HO28S21A44 (AB600372) from a thermoacidic Japanese spring field	99	Kato et al. 2011
	<i>Thermogymnomonas acidicola</i> (NR_041513)	86	Itoh et al. 2007
Clone 6A-1_CM_mar12 (KC662258) ^C	Clone HO28S9A67 (AB600348) from a thermoacidic spring field in Japan	98	Kato et al. 2011
	<i>Thermogymnomonas acidicola</i> (NR_041513)	86	Itoh et al. 2007
Clone 8C-1 (KF303810) ^{Csed1}	Clone ORCL3.3 (EF396244) from a metal-rich acidic stream in Spain	99	Rowe et al. 2007
	<i>Thermogymnomonas acidicola</i> (NR_041513)	92	Itoh et al. 2007
Clone 8C-2 (KF303811) ^{Csed1}	Clone ORCL3.3 (EF396244) from a metal-rich acidic stream in Spain	98	Rowe et al. 2007
	<i>Thermogymnomonas acidicola</i> (NR_041513)	91	Itoh et al. 2007
Clone 8C-4 (KF303812) ^{Csed1}	Clone 5A-1_CM_mar12 (KC662257)	99	This study
	<i>Thermogymnomonas acidicola</i> (NR_041513)	86	Itoh et al. 2007
Clone 8C-7 (KF303813) ^{Csed1}	Clone 5A-1_CM_mar12 (KC662257)	98	This study
	<i>Thermogymnomonas acidicola</i> (NR_041513)	86	Itoh et al. 2007
Clone 8F-6 (KF303814) ^{Csed1}	Clone DAAP3A6 (KC208503) from a stream draining in UK	94	Kay et al. 2013
	<i>Methanomassiliicoccus luminyensis</i> (HQ896499)	82	Dridi et al. 2012

Clone 8F-8 (KF303815) ^{Csed1}	Clone HO28S21A56 (AB600373) from a thermoacidic Japanese spring field	98	Kato et al. 2011
	<i>Nitrososphaera viennensis</i> (FR773158)	85	Tourna et al. 2011
Clone 38F-1 (KF303816) ^{Csed2}	Anaerobic methanogenic archaeon ET1-8 (AJ244284) from an unflooded rice fields	97	Chin et al. 1999
	<i>Ignisphaera aggregans</i> (NR_102869)	80	Niederberger et al. 2006
Clone 38F-5 (KF303817) ^{Csed2}	Clone kmc067 (HM150125) from thermal spring in Kamchatka peninsula	98	Wemheuer et al. 2013
	<i>Thermogymnomonas acidicola</i> (NR_041513)	86	Itoh et al. 2007
Clone 36F-6 (KF303818) ^{Csed2}	Clone kmc067 (HM150125) from thermal spring in Kamchatka peninsula	99	Wemheuer et al. 2013
	<i>Thermogymnomonas acidicola</i> (NR_041513)	87	Itoh et al. 2007
Clone 38F-8 (KF303819) ^{Csed2}	Clone LCDARCH18 (EU247270) from a mining impacted Coeur d'Alene river, USA	99	Rastogi et al. 2009
	<i>Ignisphaera aggregans</i> (NR_102869)	80	Niederberger et al. 2006
Clone 38F-11 (KF303820) ^{Csed2}	Clone ED1-30 (EU332089) from marine sediments	99	Park et al. 2008
	<i>Nitrososphaera viennensis</i> (FR773157)	96	Tourna et al. 2011

**NEW BACTERIAL
ISOLATES AND THEIR
RESISTANCE TO
DISSOLVED METALS**

4

Results shown in this chapter have been included in a recent paper by Falagán and Johnson 2014: *Acidibacter ferrireducens* gen. nov., sp. nov.: an acidophilic ferric iron-reducing gammaproteobacterium. *Extremophiles* 18:1067-1073.

4.1 INTRODUCTION.....	96
4.2 MATERIALS AND METHODS	99
4.2.1 CULTIVATION OF DIFFERENT ACIDOPHILIC HETEROTROPHS FROM THE GUADIANA AND THE CUEVA DE LA MORA PIT LAKES.....	99
4.2.2 CHEMOTAXONOMIC ANALYSIS.....	100
4.2.3 PHYLOGENETIC ANALYSIS.....	100
4.2.4 GRAM STAINING TEST.....	101
4.2.5 MOTILITY SCREENING.....	101
4.2.6 PIGMENT ANALYSES	101
4.2.7 SCREENING FOR CHEMOLITHOTROPHIC AND CHEMOLITHO-HETEROTROPHIC GROWTH.....	101
4.2.8 GROWTH ON ORGANIC SUBSTRATES.....	102
4.2.9 EFFECTS OF pH AND TEMPERATURE ON THE GROWTH RATE OF DIFFERENT HETEROTROPHS FROM THE PIT LAKES	102
4.2.10 IRON REDUCTION AND TESTING FOR GROWTH IN ANAEROBIC CONDITIONS.....	103
4.2.11 SENSITIVITY TO TRANSITION METALS, ALUMINIUM, ARSENIC AND SALT	104
4.3 RESULTS.....	105
4.3.1 MORPHOLOGICAL CHARACTERIZATION AND PHYSIOLOGY OF THE ISOLATES	105
4.3.2 CHEMOTAXONOMIC AND PHYLOGENETIC ANALYSIS.....	111
4.3.3 IRON REDUCTION AND TESTING FOR GROWTH IN ANAEROBIC CONDITIONS	115
4.3.4 SENSITIVITY TO TRANSITION METALS, ALUMINIUM AND SALT	116
4.4 DISCUSSION.....	119
4.4.1 ISOLATES MCF9, MCF10 AND MCF14.....	119
4.4.2 ISOLATE MCF40.....	120
4.4.3 ISOLATE MCF85.....	120
4.4.4 ISOLATE MCF105.....	120
4.4.5 METAL TOLERANCE.....	121
4.5 CONCLUSIONS.....	122
4.6 APPENDIX.....	124

Abstract

Six acidophilic bacteria (MCF9, MCF10, MCF14, MCF40, MCF85 and MCF105) belonging to three different phyla were isolated from two pit lakes at two abandoned metal mines in the Iberian Pyrite Belt mining district, south-west Spain. One of them (MCF85) was shown to be distantly related to all characterized prokaryotes, and to be the first representative of a novel genus and species; amongst the others, three belong to the same genus and the other two belong to two different genera. Five of the isolates are acidophiles (pH growth limits from 2.3 to 5.2) and one of them is acid-tolerant (pH growth limits from 3.4 to 8.4). Only organic electron donors were used by the isolates, which were confirmed to be obligate heterotrophs. Isolate MCF105 is an obligate anaerobe, whilst isolate MCF85 is a facultative anaerobe and the others are obligate aerobes. Isolates MCF9, MCF10, MCF14 and MCF85 catalysed the reductive dissolution of the ferric iron mineral schwertmannite when incubated under micro-aerobic or anaerobic conditions (only isolate MCF85), causing the culture media pH to increase. The aerobic heterotrophs grew under the presence of high metal concentration, e.g. isolate MCF85 grew in the presence of >100 mM ferrous iron or aluminium. The most toxic metal for all the isolates was copper. The majority of the isolates were much more tolerant to As(V) than to As(III) except isolate MCF105, which grew at 10 mM As(V) and 10 mM As(III). Only isolate MCF85 presented evidences of extracellular iron accumulation.

4.1 INTRODUCTION

Extremely acidic environments, generally regarded as those having pH values of less than 3.0, can be populated by a wide diversity of prokaryotic and eukaryotic microorganisms (Johnson 2009) and may occur naturally or as a result of human activities. Most widespread among the latter are environments either generated or severely affected by mining of metals and coal (Nordstrom and Alpers 1999, Younger et al. 2002, Blowes et al. 2003). Acid mine drainage streams, pit lakes and tailings are common relic features of abandoned mines and may persist for many (often >100) years after mine closure. The acidity of these water bodies derives from the microbially-accelerated oxidative dissolution of pyrite and other sulphide minerals (as explained previously in Chapter 1, Banks et al. 1997, Vera et al. 2013). The sulphuric acid generated greatly enhances the solubility of many metals that are also released from the oxidized sulphides and other minerals, causing such water bodies to be harmful or lethal to most forms of life.

Primary production in extremely acidic environments is mediated by both eukaryotic micro-algae (Aguilera et al. 2007) and chemolithotrophic prokaryotes (Kimura et al. 2011). The latter use primarily ferrous iron and/or reduced forms of sulphur, both of which occur in pyrite and some other sulphide minerals, as electron donors, and include some of the most widely studied of all acidophiles (*Acidithiobacillus* spp., *Leptospirillum* spp. etc.). In contrast, other species of acidophiles are obligate heterotrophs (e.g. most *Acidiphilium* spp., and *Acidocella* spp.) while a third group (including *Sulfobacillus* spp. and *Acidiphilium acidophilum*) can both fix CO₂ and utilize organic carbon. One characteristic that appears to be widespread among acidophiles is the ability to use ferric iron as an alternative electron acceptor to oxygen when these are grown under anaerobic or micro-aerobic conditions. All iron-oxidizers that can utilize an alternative electron donor to ferrous iron appear to do this (e.g. *Acidithiobacillus* (*At.*) *ferrooxidans*, *At. ferrivorans* and *At. ferridurans*) as can many obligately heterotrophic species, including *Acidiphilium* and *Acidocella* spp. (both α -proteobacteria), the γ -proteobacterium *Metallibacterium scheffleri* (Ziegler et al. 2013), and *Acidobacterium capsulatum* and related species (Coupland and Johnson 2008). The reason for ferric iron respiration being particularly widespread among acidophiles appears to be: (i) ferric iron is often abundant and more bio-available at low pH than in near-neutral pH environments; (ii) the redox potential of the ferrous/ferric couple is far more positive at pH ~2 (often quoted at +770

mV, though it is ~+690~+730 mV in sulphate-rich acidic liquors, due to complexing of iron; Johnson et al. 2012) than at circum-neutral pH.

Acidophilic heterotrophs are widespread throughout acidic (pH <3) environments (Johnson 1998, Johnson and Hallberg 2003, Hallberg 2010), including rivers or streams affected by acid mine drainage (Walton and Johnson 1992, McGinness and Johnson 1993, Hallberg and Johnson 2003, Rowe et al. 2007, González-Toril 2009, 2011, Hao et al. 2012) or acid pit lakes (Gyure et al. 1987, Kamjunke et al. 2005, Sánchez-España et al. 2007, Porsch et al. 2009, Santofimia et al. 2013, Falagán et al. 2014) and belong to different bacterial phylogenetic groups. The majority of these acidophilic bacteria are mesophiles and can assimilate a wide range of organic substrates.

Acidophiles are tolerant to high metal concentration. Some metals are necessary for biochemical processes (e.g. Fe, Mn, Ni, Cu), but excessive quantities can cause negative effects (e.g. oxidative stress, protein activity inhibition). Microorganisms have developed different strategies to minimize these effects, including processes leading to the immobilization of metals such as biosorption, intracellular accumulation or the production of metal-binding biomolecules (Gadd et al. 2004). A neutrophilic sulphate-reducing bacterium was found to bioprecipitate chromium (III) phosphate on the cell surface (Goulhen et al. 2006), though sometimes these mechanisms might lead to cell death. Metal sensitiveness varies amongst acidophiles (Dopson et al. 2003). For example different species of the same genus (*Acidocella* sp.) are able to grow at Al³⁺ concentrations of 200-500 mM (Jones et al. 2013). Other examples are *At. ferrooxidans*, *At. ferrivorans* and *At. ferridurans* which can grow at 300 mM Al³⁺ (Hedrich and Johnson 2013b). Not much is known about the mechanisms by which acidophiles tolerate the presence of high metal concentrations, which are a current topic of discussion amongst scientists. Microorganisms have developed different mechanisms to avoid the toxicity produced by the presence of high metal concentrations such as (i) efflux metals out of the cell, (ii) enzyme conversion, (iii) intra- or extracellular sequestration, (iv) exclusion by a permeability barrier, and (v) reduction in sensitivity of cellular targets (Dopson et al. 2003). The majority of the investigations have been done in the most studied acidophile *At. ferrooxidans*, Dopson et al. (2003), for instance, describes bacterial mechanisms to reduce the toxicity of different metals in bioleaching processes. The authors have found three different types of efflux system (one for As(III), a different one for cadmium and another for copper), another possible mechanisms consist in keeping the metal (nickel) bound to the cell surface, and the reduction of the metal cation to a less toxic form, e.g.

Hg(II) is reduced to Hg(0) which volatilizes out of the cell. However, these authors could not clarify the zinc resistance mechanism, which is believed to be chromosomally encoded (Dopson et al. 2003). The toxicity of other common metals in acidic environments (As, Fe or Al) has also been studied in other works. As-biomineralization has been described and intensively studied by transmission electron microscopy (TEM) in an acid mine drainage site in France (Leblanc et al. 1996, Casiot et al. 2003, 2004, Bruneel et al. 2006, Benzerara et al. 2008, Egal et al. 2009). Although ferric iron is the most abundant metal in acidic environments, the resistance mechanisms of acidophiles to this metal are not clear. Recently, a bacterially mediated precipitation of Fe-carbonates has been described in *Acidiphilium* sp. PM, which was isolated from an acidic river in Spain (Sánchez-Román et al. 2014). Aluminium toxicity has not been studied at molecular level although there are some papers regarding the bacterial tolerance to this metal (Kawai et al. 2000, Fischer et al. 2002, Ojumu et al. 2007, Blight and Ralph 2008, Watkin et al. 2009). In Meier et al. (2012) a bacterially mediated Al precipitation was detected in sulphate reducing bacteria cultures.

During a study of the microbiology and biogeochemistry of two acidic pit lakes (Cueva de la Mora and Guadiana; Chapter 2), different bacteria isolates were found to be moderately distantly or distantly related (90-97% 16S rRNA gene similarity) to all currently classified bacteria (Chapter 3; Falagán et al. 2014). The majority of the isolates (MCF9, MCF10, MCF14, MCF40, MCF85) were shown to be obligately acidophilic and one of them acid-tolerant (MCF105). All of the isolates were heterotrophic and four of the isolates (MCF9, MCF10, MCF14 and MCF85) were observed to be able to catalyse the dissimilatory reduction of ferric iron under micro-aerobic conditions. A total of four different species (two isolates appeared to be the same species) are described in this work, "*Acidicapsa ferrireducens*", "*Acidicapsa acidophila*", "*Granulicella acidophila*", and "*Clostridium acididurans*", in addition to a novel genus and species (*Acidibacter ferrireducens*). Here we describe in full the characteristics of these previously unknown acidophilic bacteria and the responses of these isolates to the presence of different common metals found in the acidic environments from which these bacteria were isolated.

4.2 MATERIALS AND METHODS

4.2.1 CULTIVATION OF DIFFERENT ACIDOPHILIC HETEROTROPHS FROM THE GUADIANA AND THE CUEVA DE LA MORA PIT LAKES

Different strains of heterotrophs were isolated from the Guadiana and from Cueva de la Mora pit lakes. These isolates were obtained from the water column of Cueva de la Mora and Guadiana (Table 4.1) in March 2012, except for MCF105, which was obtained from the Cueva de la Mora sediments collected in February 2012.

Table 4.1. Isolates, lake and depth origin, water pH, redox (E_H) and dissolved oxygen (DO) where the isolates were taken. CM, Cueva de la Mora pit lake; G, Guadiana pit lake; CMsed, 38 m depth Cueva de la Mora sediments.

Isolates	Lake	Depth (m)	pH	E_H	DO (%)
MCF9	CM	3	2.7	790-850	100
MCF10	CM	10.5	3.0	290-600	2
MCF14	G	2.9	3.0	720-800	100
MCF40	G	15	2.8	650-700	20
MCF85	G	15	2.8	650-700	20
MCF105	CMsed	38	4.5/4.0*	310/195*	0

*, sediments interstitial water.

Different colour and shape colonies (Fig.4.1) subsequently identified as different isolates grew aerobically, except for the MCF105 isolate which grew anaerobically, on yeast extract overlay plates (Ye4o), with a pH of ~3.5. The isolates were purified by repeated single colony re-isolation, and confirmed as being axenic with terminal restriction enzyme fragment length polymorphism analysis (T-RFLP), using three restriction enzymes (Table 4.2) that differentiate between known species of acidophilic bacteria (Kay et al. 2013).

The isolates were transferred into a liquid medium and subsequently maintained in these media, with short-term (up to two weeks) storage of cultures at 4°C. Isolates MCF9, MCF10, MCF14, MCF40, MCF85 and MCF105 were grown in yeast extract (YE) medium at a set pH (MCF9, MCF10, MCF14 and MCF40 in 0.01% YE, pH 4.0; MCF85 in 0.002% YE, pH 3.5; and MCF105 in 0.02% YE, pH 4.5). Glucose (5 mM) was used as carbon source in all the cultures.

Table 4.2. Restriction enzymes cutting sites on the 16S rRNA gene for the different isolates.

	Haelll	CfoI	AluI
MCF9	214 ± 2	93 ± 2	222 ± 2
MCF10	214 ± 2	93 ± 2	222 ± 2
MCF14	214 ± 2	93 ± 2	222 ± 2
MCF40	242 ± 2	93 ± 2	154 ± 2
MCF85	255 ± 2	209 ± 2	236 ± 2
MCF105	219 ± 2	226 ± 2	233 ± 2

4.2.2 CHEMOTAXONOMIC ANALYSIS

Chromosomal DNA for G+C content of isolate MCF85 was extracted with Blood & Cell Culture DNA Midi Kit (QIAGEN) following the manufacturer's instructions, and G+C content was determined by reverse-phase high performance liquid chromatography (HPLC) using a Nucleosil 100-5 C18 column (Macherey-Nagel) and purified non-methylated DNA of lambda phage (Sigma) as a standard, following the protocol described by Tamaoka and Komagata (1984). Freeze dried cells of glucose/yeast-grown MCF85 were sent to the Identification Service of the Leibniz-Institute DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) for analysis of fatty acids, polar lipids and respiratory quinones.

4.2.3 PHYLOGENETIC ANALYSIS

The 16S rRNA gene of all the isolates was amplified by PCR, using the primers 27F (5'-3' AGAGTTTGATCMTGGCTCAG; Lane 1991) and 1387R (5'-3' GGGCGGWTGTACAAGGC; Marchesi et al. 1998). The gene sequence obtained was analysed and compared with those in the NCBI database using BLASTN online software (National Centre of Biotechnology Information, NCBI). The 16S rRNA genes of the different isolates and other selected iron reducers and acidophilic heterotrophs were aligned using Clustal X (Larkin et al. 2007). Phylogenetic trees were generated using neighbour-joining analysis with full-length sequences after removing gaps and highly variable positions. Bootstrap analyses were carried out on 1000 replicate input data sets. The trees were rooted with the 16S rRNA gene sequence of the iron-oxidizing acidophile *Leptospirillum ferrooxidans* (GenBank accession number X86776).

4.2.4 GRAM STAINING TEST

A drop liquid culture was placed on a slide. The sample was dried by passing it through a flame. A drop of crystal violet was added and left for a minute and then, the staining solution was washed out with water. Iodine solution was added and left for another minute, and gently rinsed with water. As a final step, alcohol was added to the slide to allow decolourisation and air drying. Gram-positive bacteria appeared as purple while the Gram-negative bacteria appeared pink.

4.2.5 MOTILITY SCREENING

Motility studies were performed with a transmission electron microscope (TEM) Philips CM120 at the SGiker services in the Basque Country University. Isolates were grown in liquid cultures at the appropriate medium for each isolate (Table 4.3). Fresh cultures were fixed with 2% glutaraldehyde in Sorensen buffer (0.1 M); the staining was made by placing the samples in uranyl acetate for 1 minute.

4.2.6 PIGMENT ANALYSES

Bacterial suspensions were centrifuged at 13,000 g; the resulting pellet was re-suspended in 3 mL acetone-methanol solution and placed inside an ultrasound bath for 2 hours at 4°C. The samples were centrifuged at maximum speed (13,000 g) and the supernatant was transferred into clean microfuge tube. The spectral scan was performed at a wavelength from 340 to 900 nm on the supernatant using a glass cuvette (method modified from Kishimoto et al. 1995).

4.2.7 SCREENING FOR CHEMOLITHOTROPHIC AND CHEMOLITHO-HETEROTROPHIC GROWTH

The ability of the different isolates to grow autotrophically or heterotrophically using ferrous iron or elemental sulphur as electron donors was tested by inoculating liquid media, amended or not with yeast extract, containing either 5 mM ferrous sulphate (pH 3.0) or 1% (w/v) elemental sulphur (pH 3.5). Parallel non-inoculated controls were also set up. Oxidation of ferrous iron was assessed by measuring changes in concentrations of ferrous iron (using the ferrozine assay; Stookey 1970) and changes in culture pH as a measure of sulphur oxidation. Growth on hydrogen was tested using the method

described by Hedrich and Johnson (2013a). Amplification of *cbbL* and *cbbM* and *nifH* genes (Table 4.3; *cbbL* gene encodes for the large subunit for type I RuBisCo (Ribulose-1,5-bisphosphate carboxylase/oxygenase); *cbbM* gene for the large subunit for type II RuBisCo; and *nifH* gene for the dinitrogenase reductase) from lysates of the different isolates was attempted using the protocol described by Johnson et al. (2009) and lysates of the autotrophic diazotroph *At. ferrooxidans* as a positive control.

Table 4.3. Sequences of the primers for specific genes: *nifH*, encodes the dinitrogenase reductase; *cbbL*, encodes the large subunit for type I Rubisco; *cbbM*, encodes the large subunit for type II Rubisco.

Primer	Sequence (5' - 3')	Reference
<i>nifH</i> 19F	GCIWTYTAYGGIAARGGIGG	Ueda et al. 2005
<i>nifH</i> 407R	AAICCRCCRCIAIACIACRTC	Ueda et al. 2005
<i>cbbL</i> F	CGGCACSTGGACCACSGTSTGGAC	Alfreider et al. 2003
<i>cbbL</i> R	GTARTCGTGCATGATGATSGG	Alfreider et al. 2003
<i>cbbM</i> F	GGCACCATCATCAAGCCCAAG	Alfreider et al. 2003
<i>cbbM</i> R	TCTTGCCGTAGCCCATGGTGC	Alfreider et al. 2003

4.2.8 GROWTH ON ORGANIC SUBSTRATES

The ability of the isolates to use organic substrates was tested by growing them in the usual media (section 4.2.1) amended with various organics compounds. Different concentrations of substrates were used to roughly equalize their carbon-equivalents (i.e. 5 mM for C₆ substrates, 10 mM for C₃ substrates etc.). Replicate universal bottle cultures containing 10 mL of test medium were inoculated and incubated (shaken) at 30°C. Growth was determined by measuring the optical densities of cultures at 600 nm (OD₆₀₀) after one (isolates MCF85) or two (the rest of the isolates) weeks and compared with those obtained in both negative controls (no organic substrate added) and positive controls (5 mM glucose added).

4.2.9 EFFECTS OF pH AND TEMPERATURE ON THE GROWTH RATE OF DIFFERENT HETEROTROPHS FROM THE PIT LAKES

To determine its pH and temperature limits for growth, all the isolates (MCF9, MCF10, MCF14, MCF40, MCF85 and MCF105) were cultured in shake flasks.

Incubation temperatures ranged from 8 to 40°C at different pH in which was detected the best growth of each isolate, and the pH values of the liquid medium varied between 2.0 and 5.0 (for flasks incubated at 30°C), except for MCF105 which varied between 3.0 and 8.0. To avoid any pH changes resulting from autoclaving the medium and adding the bacterial inoculum, the pH-adjusted complete media were filter-sterilized and only small volumes (200 µL) of inoculum were added to 50 mL culture aliquots.

To determine the optimal growth conditions, isolate MCF85 was grown in a 1.5 L (working volume) bioreactor (Electrolab Ltd., UK) fitted with pH, temperature and aeration control. Isolate MCF85 was grown under aerobic condition and stirred at 100 rpm. To determine optimum pH, the temperature was maintained at 30°C and pH fixed at set values between 2.5 and 4.5, samples were withdrawn at regular intervals and optical densities at 600 nm (OD_{600}) were measured. Culture doubling times were determined from semi-logarithmic plots of OD_{600} values against time. To determine the optimum temperature for growth, the bioreactor pH was maintained at 3.5 (by automated addition of 0.1 M H_2SO_4 or 0.1 M NaOH) and temperature set at between 28 and 45°C.

4.2.10 IRON REDUCTION AND TESTING FOR GROWTH IN ANAEROBIC CONDITIONS

To determine whether all the isolates could catalyse the dissimilatory reduction of ferric iron, experiments were carried out in the corresponding media (section 4.2.1) grown in both micro-aerobic and anoxic conditions in the presence of the ferric iron mineral schwertmannite (stoichiometry $Fe_8O_8(SO_4)_x(OH)_y \cdot nH_2O$). Ferric iron is not soluble at the pH range of any isolate, and schwertmannite is a major ferric iron mineral that forms in ferruginous sulphate waters of pH ~3-5 (Bigham et al. 1996). Schwertmannite was produced using the method described by Schwertmann and Cornell (2000) and dried at 37°C. One hundred mg of dried mineral was added to triplicate flasks containing 18 mL of 10 mM glucose and 0.002% (MCF85), 0.01% (MCF9, MCF10 and MCF14) or 0.02% (MCF105) yeast extract medium (pH 3.0, pH 4.0 for MCF105) and inoculated with 2 mL of an active culture. A non-inoculated control was set up in parallel as a control. The flasks were placed in sealed 2.5 L jars within which micro-aerobic and anaerobic conditions were generated using the CampyGen® sachets and AnaeroGen® sachets (Oxoid, UK) respectively. Samples were withdrawn on a regular basis over 10 to 21 days, and ferrous iron concentrations determined using the ferrozine

assay (Stookey 1970). The pH of the cultures was measured at the start and at the end of the experiment.

A longer-term (30 day) experiment with the isolate MCF85 was set up in parallel using either 50 or 100 mg of dried mineral in cultures and mineral-free culture that were incubated under anoxic conditions using AnaeroGen[®] sachets (Oxoid, UK). Ferrous iron concentrations and pH were measured as before, and planktonic cells were enumerated using a Thoma counting chamber.

4.2.11 SENSITIVITY TO TRANSITION METALS, ALUMINIUM, ARSENIC AND SALT

The ability of the isolates to tolerate elevated concentrations of some transition metals (Mn^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+}), aluminium, ferrous iron, and arsenic (As(III) and As(V)) was tested by adding different concentrations of the metals (as their corresponding sulphate salts), sodium arsenite or sodium arsenate to the growing media. The cultures were incubated for one week and OD_{600} values were compared to those of metal-free control cultures. Arsenic (III) and arsenic (V) tolerance was compared in two other acidophilic heterotrophic bacteria (*Acidiphilium cryptum* strain SJH, and *Acidocella* (*Ac.*) *aromatica*^T). Salt (NaCl) tolerance was tested by supplementing glucose/yeast extract medium with 50-500 mM NaCl, and magnesium sulphate-supplemented cultures served as controls.

Intra- and extracellular metal accumulation was searched in three of the six isolates (MCF9, MCF40, MCF85) at similar metal concentration to their limit concentration for growth (Table 4.4). Metal accumulation was compared with *Ac. aromatica*^T, which also tolerates high iron and aluminium concentrations (Jones et al. 2013). The three isolates and *Ac. aromatica*^T were grown in 0.02% yeast extract medium and supplemented with 10 mM glucose (10 mM fructose for *Ac. aromatica*^T); different Fe(II), aluminium and As (V) (only MCF85) concentrations (Table 4.4) were added to separated cultures, metal-free cultures served as controls. Fresh cultures were placed on a 300 mesh copper grid and air-dissected before TEM study. Other set of samples were embedded into an epoxy resin following the protocol given in the appendix, the resin blocks were cut with a microtome LEICA Ultracut UCT and the slides placed on a copper grid. Samples were looked under a Philips CM200 microscope equipped with an EDS detector in the Basque Country University (SGiker services).

Table 4.4. Metal concentration under the different isolates were grown for the posterior preparation of epoxy resins and direct observation of the liquid cultures under the transmission electron microscope.

	Fe(II)	Al	As(V)
	mM	mM	mM
MCF9	10	100	
MCF40	10	50	
MCF85	160	110/50	0.1/1
<i>Ac. aromatica</i>^T	120	100/200	

4.3 RESULTS

4.3.1 MORPHOLOGICAL CHARACTERIZATION AND PHYSIOLOGY OF THE ISOLATES

Isolates MCF9 and MCF14 grew as pink-tinged colonies on Ye4o solid medium, and isolate MCF10 grew as white colonies in the same medium (Fig. 4.1). The three isolates needed yeast extract for growth. Cells were rod-shaped and isolate MCF10 formed long chains of cells, and MCF9 (Fig. 4.2) and MCF14 cells were motile. These isolates grew in liquid media but did not grow in the absence of yeast extract (minimum requirement was 0.01%). MCF9 and MCF14 had β -carotene as a pigment (Fig. 4.3). Isolates MCF40 and MCF85 (Fig. 4.1) grew as off-white and white/green-tinged colonies, respectively, on Ye3o solid medium. These isolates grew slowly in liquid medium containing glucose as sole carbon and energy source, but growth was greatly enhanced by the addition of yeast extract (at 0.002% and above). Cells were non-motile and rod-shaped. Isolate MCF105 grew as white colonies with snowflake-like shape in Ye4o plates (Fig. 4.1). This isolate grew in liquid medium with a minimum requirement of 0.02% yeast extract. Cells were rod-shaped and motile (Fig. 4.2). The dimensions of the isolates were similar except for MCF105, which was the longest rod (Table 4.5). Isolates MCF9, MCF14, MCF10, MCF40 and MCF85 cells stained Gram-negative, while MCF105 cells stained Gram-positive.

No oxidation of inorganic electron donors (ferrous iron, sulphur and hydrogen) by any of the isolates was observed. Growth did not occur in the absence of an organic substrate, and RuBisCo genes were not amplified from cells lysates in any of the isolates,

while *nifH* genes were amplified just in the isolate MCF40 and MCF105. It was concluded therefore that all these isolates are obligately heterotrophic bacteria.

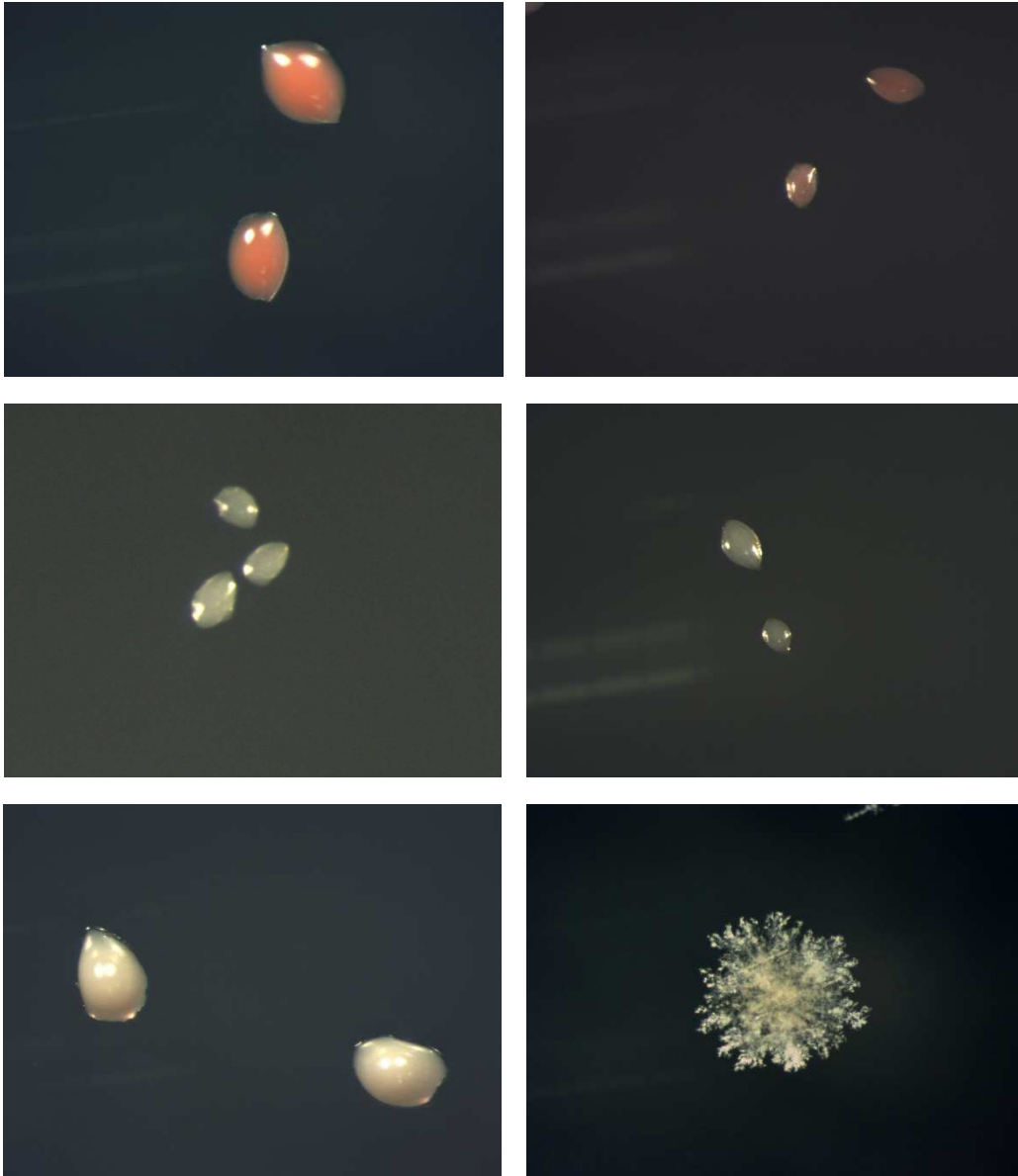


Figure 4.1. Colonies of the different isolates growing on Ye4o plates looked under the binocular microscope at different magnifications (10-20x): MCF9 (left top); MCF14 (right top); MCF10 (left middle); MCF40 (right middle); MCF85 (left bottom); MCF105 (right bottom).

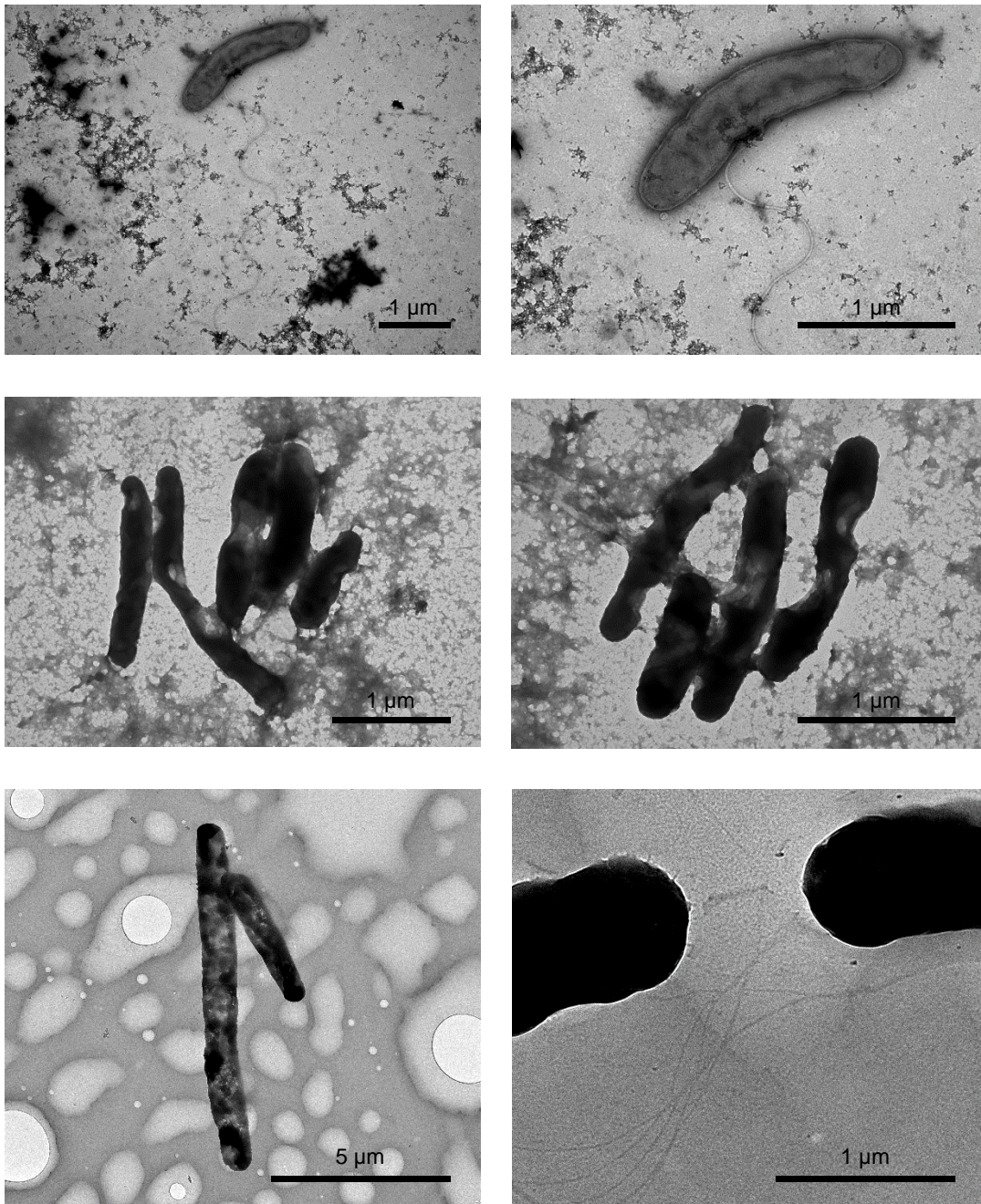


Figure 4.2. Pictures taken under the transmission electron microscope of liquid cultures of isolates MCF9 (top), MCF85 (middle) and MCF105 (bottom).

Table 4.5. Cell dimensions, presence/absence of flagella and pigmentation of the different isolates.

	Length (μm)	Motility	Pigment
MCF9	1.8-2.2	Lateral flagella	Beta-carotene
MCF10	n.d.	n.d.	No
MCF14	1.8-2.2	Flagella	Beta-carotene
MCF40	0.5-1.5	No	No
MCF85	1.5-2.5	No	No
MCF105	6-9	Polar flagella	No

n.d., not determined.

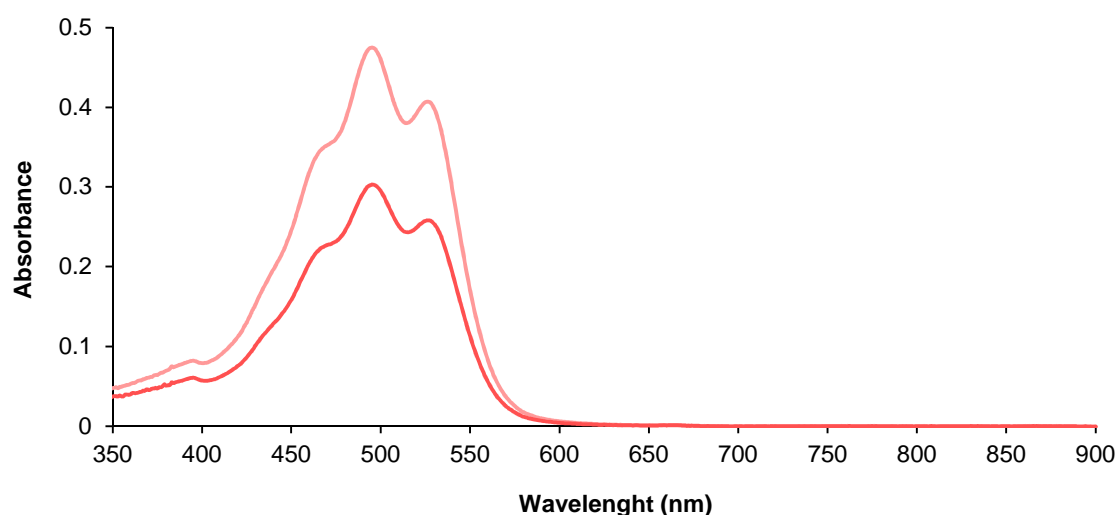


Figure 4.3. Pigment scan of the isolates MCF9 (dark pink) and MCF14 (light pink) showing the typical profile of β -carotenes with three peaks between 450 and 550 nm wavelength.

The different isolates grew on a limited range of organic compounds (Table 4.6). The majority of the small molecular weight organic compounds tested inhibited the growth of the isolates at the concentrations used except for the isolate MCF105, whose growth was not inhibited. MCF9 and MCF14 presented the same responses to the different substrates tested, and not many differences with MCF10, which was the only isolate studied to be able to grow on lactic acid. Isolate MCF40 did not use any alcohol, but used a wide range of substrates. Isolate MCF85 grew on a limited range of organic compounds, including some hexoses (though not pentoses or methyl pentoses) and some disaccharides.

Table 4.6. Utilization of organic compounds by the characterized isolates. OD₆₀₀ values were measured after one week and compared with those of positive controls (glucose/yeast extract) and base-line controls (yeast extract only). Key: (++) OD₆₀₀ values higher than positive control; (+) OD₆₀₀ values between similar to that of positive control; (-) OD₆₀₀ values similar to positive control; (I) OD₆₀₀ values lower than negative control (partial inhibition of growth); (C) OD₆₀₀ <0.01 (complete inhibition of growth).

Substrate	Conc. (mM)	MCF9	MCF10	MCF14	MCF40	MCF85	MCF105
Glucose	5	+	+	+	++	++	++
Sorbose	5	-	-	-	-	+	-
Galactose	5	+	+	+	+	-	-
Mannose	5	+	+	+	++	-	++
Fructose	5	+	+	+	+	+	-
Rhamnose	5	+	+	+	++	-	+
Fucose	5	-	-	-	+	-	-
Arabinose	5	-	-	-	-	-	+
Xylose	5	+	+	+	++	-	+
Ribose	5	-	+	-	+	-	-
Sucrose	5	+	++	+	++	++	++
Maltose	5	+	+	+	++	++	++
Lactose	5	+	+	+	++	+	+
Trehalose	5	+	+	+	+	++	++
Cellobiose	5	+	+	+	++	+	++
Glucosamine	5	-	-	-	++	+	+
Glucuronic acid	5	I	I	I	++	-	C
Mannitol	5	-	-	-	-	+	+
Glycerol	10	+	+	+	-	+	++
Ethanol	15	-	-	-	-	-	-
Methanol	20	-	-	-	-	-	-
Glycine	5	n.d.	n.d.	n.d.	n.d.	I	-
Glutamic acid	5	C	C	C	I	C	-
Arginine	5	n.d.	n.d.	n.d.	n.d.	+	-
Formic acid	5	C	C	C	I	I	-
Acetic acid	1-10	C	C	C	I	C	-
Citric acid	5	C	C	C	I	C	-
Lactic acid	5	C	+	C	I	C	-
Pyruvate	5	C	C	C	I	n.d.	-
Propionate	5	C	C	C	I	n.d.	-
Benzyl alcohol	5	-	-	-	-	-	I
Benzoate	5	C	C	C	I	I	I
Phenol	5	-	-	-	-	-	I

n.d., not determined.

Conc., concentration.

The effects of temperature and pH on culture doubling times (t_d 's) of isolate MCF85 are shown in Fig. 4.4. Temperature and pH optima for growth were between 30 and 35°C and pH 3.5 and 4.0, respectively. Under optimal conditions (pH 3.5 and 35°C), the culture doubling time of isolate MCF85 was about 75 minutes.

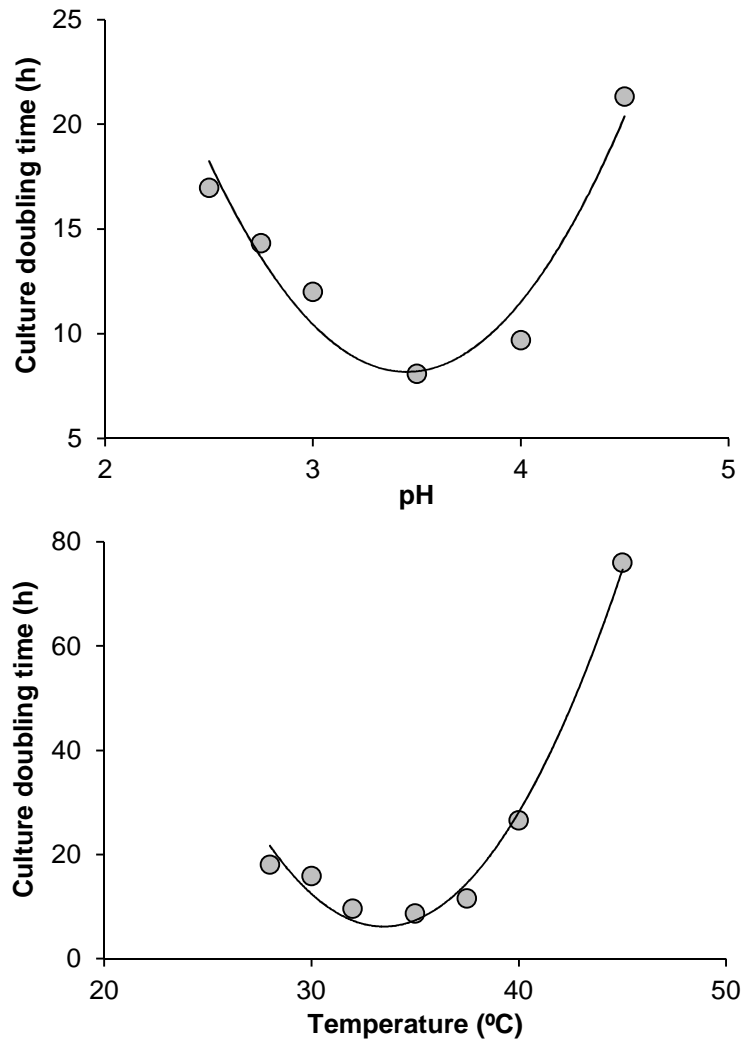


Figure 4.4. Effect of pH (top; at a constant temperature of 30°C) and temperature (bottom; at a constant pH of 3.5) on the growth of isolate MCF85.

The majority of the isolates did grow between pH 2.3 to 5.3, indicating that they are moderately acidophilic microorganisms, except for MCF105 which, being able to grow from 3.4 to 8.4, can be categorized as acid-tolerant (Table 4.7). The isolates MCF85 and MCF105 did not grow at very high or low temperature (Table 4.7), and can therefore be

categorized as mesophiles. However, isolates MCF9, MCF10, MCF14 and MCF40 were able to grow at 10°C.

Table 4.7. Temperature and pH limit growth. *Isolate MCF85 did not grow at 8°C or at **45°C.

	Lower pH limit	Higher pH limit	Growth at 10°C	Growth at 40°C
MCF9	3.2	5.2	+	-
MCF10	2.3	5.1	+	-
MCF14	3.2	5.2	+	-
MCF40	2.3	5.3	+	-
MCF85	2.5	4.5	+*	+**
MCF105	3.4	8.4	-	-

4.3.2 CHEMOTAXONOMIC AND PHYLOGENETIC ANALYSIS

The chromosomal DNA of isolate MCF85 had a G+C content, determined by HPLC, of 70.5±0.7 mol %. The major fatty acids of cells grown in glucose/yeast extract medium were C_{12:0} 2-OH, iso-C_{14:0}, C_{14:0}, iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0}, C_{16:0}, methyl-C_{16:0}, iso-C_{17:0}, anteiso-C_{17:0}, C_{18:0}. Polar lipids identified were five phospholipids, glycolipid, phosphatidylethanolamine and aminolipids. Ubiquinone-8 (Q-8) was the only respiratory quinone detected. BLAST searches using 16S rRNA gene sequences from MCF85 culture indicates that similar bacteria have been detected in clone banks of samples in different global locations, including sediments in the Tinto river, Spain (Sánchez-Andrea et al. 2011), acid mine drainage in copper mines in China (Yin et al., 2008), a coal mine in Germany (Lu et al. 2010) and tailings sediments in the Atacama, desert, Chile (Korehi et al. 2013). The closest cultivated relative (which shares 90% similarity of its 16S rRNA gene) is *Steroidobacter denitrificans*, a steroidal hormone-degrading bacterium isolated from anoxic digested sludge (Fahrbach et al. 2008). Both MCF85 and *Steroidobacter denitrificans* probably belong to the same family, as indicated in Fig. 4.5.

BLAST searches using 16S rRNA gene sequences from MCF9, MCF10 and MCF14 have shown that the three isolates share 99% similarity among themselves and other isolates found in Cantareras adit, Spain (Rowe et al. 2007), in Mynydd Parys copper mines in Anglesey, Wales, (Hallberg et al. 2006), Wheal Jane tin mine in Cornwall, England (Hallberg and Johnson 2003), and in Vale Copper Cliff Central Tailings facility,

Brazil, (Auld et al. 2013). Also, the gene sequences of this three isolates present 97% similarity with different species of the genus *Acidicapsa* (*Ad.*; *Ad. ligni* and *Ad. borealis*) from acidic *Sphagnum* peat and wood under decay of the fungus *Hyploma fasciculare*, (Kulichevskaya et al., 2012), which means that belong to the same genus but are different species as they cluster separately from the other *Acidicapsa* spp (Fig. 4.6).

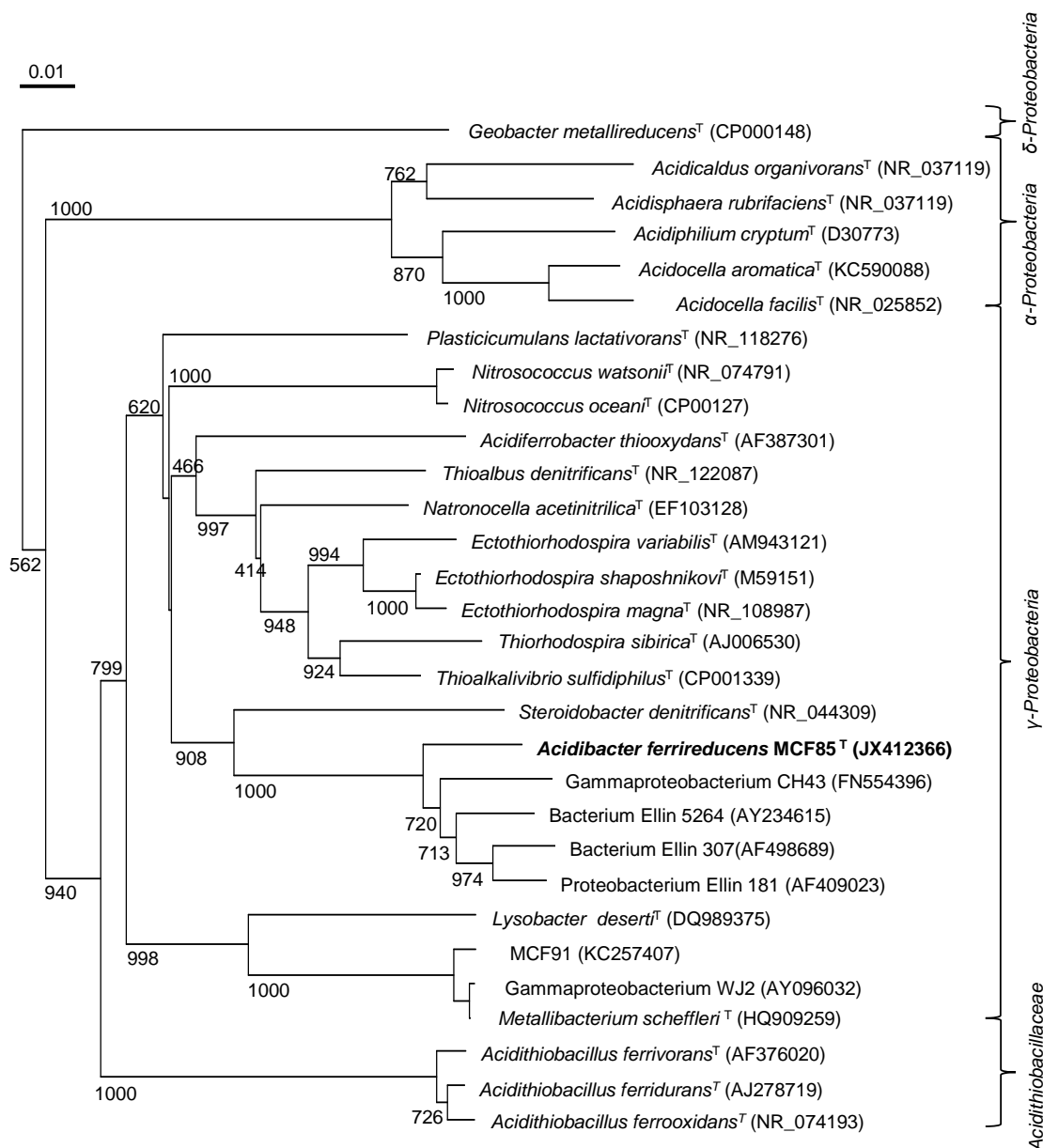


Figure 4.5. Phylogenetic tree showing the relationship of isolate MCF85 to closely-related γ -Proteobacteria and other Proteobacteria and Acidithiobacillaceae species. Bootstrap values are given at the respective nodes. The scale bar represents 0.01% sequence divergence. The tree was rooted with the 16S rRNA gene sequence of *Leptospirillum ferrooxidans*^T (X86776).

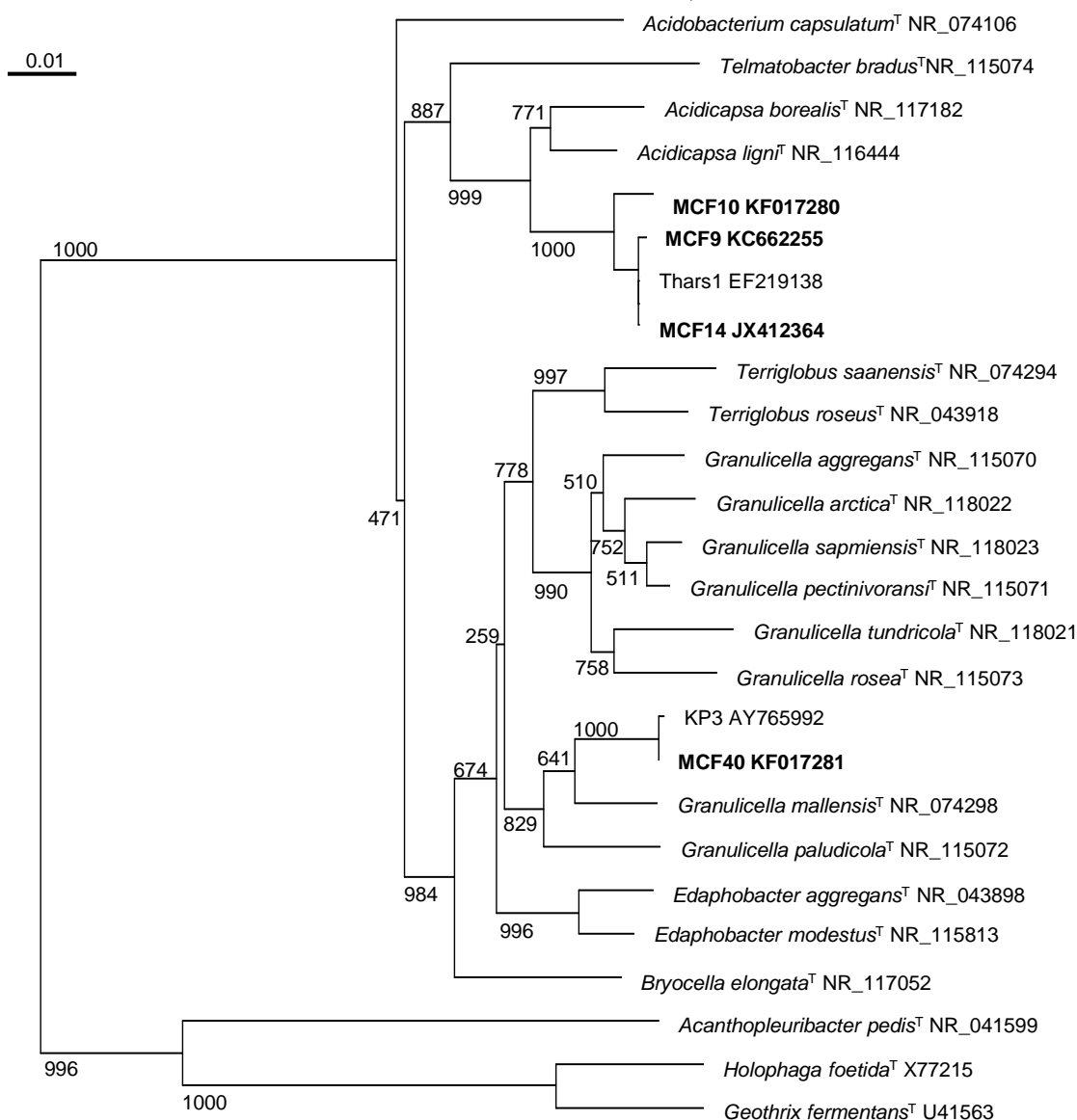


Figure 4.6. Phylogenetic tree showing the relationship of isolates MCF9, MCF10, MCF14 and MCF40 to closely-related *Acidobacteria*. The scale bar represents 0.01% sequence divergence. The tree was rooted with the 16S rRNA gene sequence of *Leptospirillum ferrooxidans*^T (X86776).

BLAST searches using 16S rRNA gene sequences from MCF40 have shown that similar bacteria have been detected in a clone bank of Río Tinto (García-Moyano et al. 2012). Another similar isolate was obtained from Mynydd Parys copper mines in Anglesey (Wales) (Hallberg et al. 2006). The closest cultivated relatives are *Granulicella paludicola* and *Granulicella mallensis* (97% similarity with both bacterial species, Pankratov and Dedysh 2010 and Mannistro et al. 2012, respectively) suggesting that the isolate MCF40 belongs to the same genus but different species (Fig. 4.6).

BLAST searches using 16S rRNA gene sequences from MCF105 have shown that similar bacteria have not been detected previously. The closest cultivated relatives are *Clostridium* (*C.*) *carboxidivorans* and *C. drakei* (96% similarity with both species, Liou et al. 2005) suggesting that MCF105 isolate belongs to this genus but is a new species (Fig. 4.7).

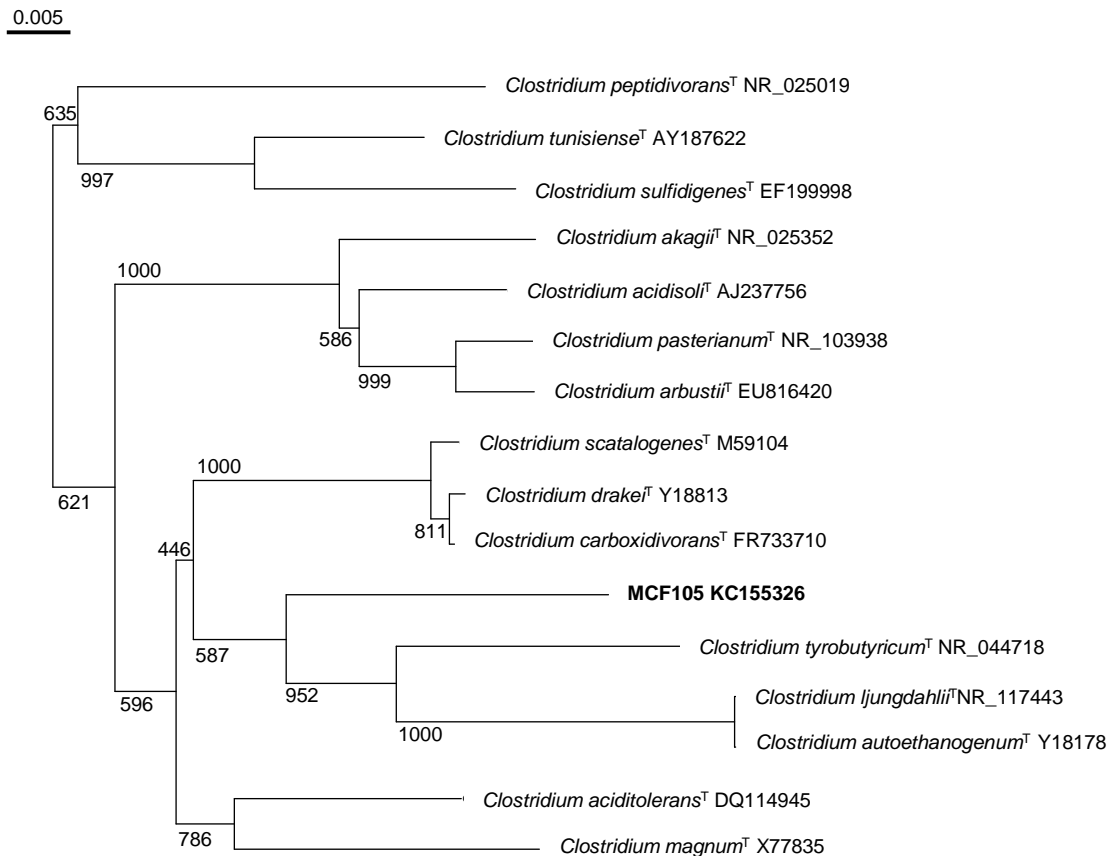


Figure 4.7. Phylogenetic tree showing the relationship of isolate MCF105 to closely-related *Clostridiales*.

Bootstrap values are given at the respective nodes. The scale bar represents 0.005% sequence divergence. The tree was rooted with the 16S rRNA gene sequence of *Leptospirillum ferrooxidans*^T (X86776).

In this work, isolate MCF85 has been designated as the type strain of the novel genus and species *Acidibacter ferrireducens*. It has been deposited in the *Netherlands Culture Collection of Bacteria* (Utrecht, The Netherlands) and the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ; Braunschweig, Germany) and has the accession numbers NCCB 100460 and DSM 27237, respectively. The isolate MCF9 has been deposited in the DSMZ under the accession number DSM 28997. The other

isolates have been also deposited in the DSMZ and are in processes of being assigned their accession numbers.

4.3.3 IRON REDUCTION AND TESTING FOR GROWTH IN ANAEROBIC CONDITIONS

Isolates MCF9, MCF10, MCF14 and MCF85 catalysed the dissimilatory reductive dissolution of the ferric iron mineral schwertmannite when incubated under micro-aerobic and, just isolate MCF85, under anaerobic conditions (Fig. 4.8 and 4.9).

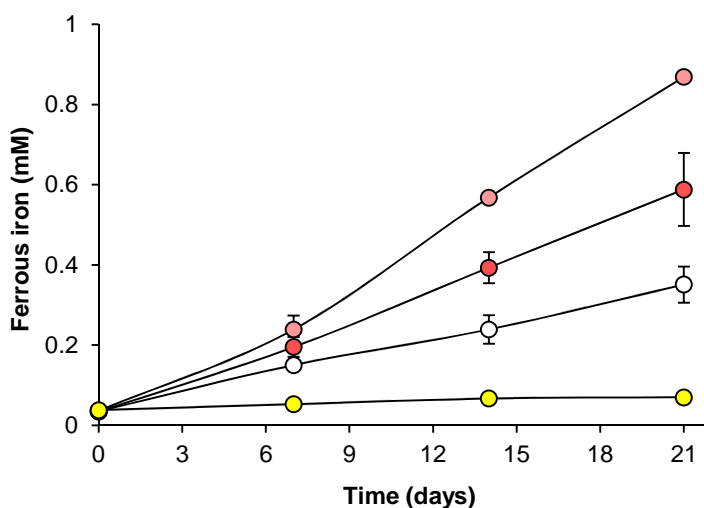
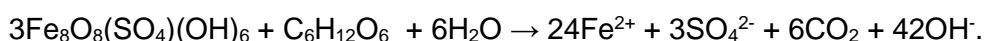


Figure 4.8. Reductive dissolution of schwertmannite by isolates MCF9 (●), MCF10 (○), MCF14 (●) and MCF40 (●), which does not show iron reduction, in cultures containing 100 mg of mineral incubated under micro-aerobic conditions.

Rates of iron reduction by MCF85 were much greater than to those of the other isolates, especially when cultures were incubated under a micro-aerobic rather than in anoxic conditions. The pH of cultures increased (e.g. from 3.0 to a mean value of 4.1 in micro-aerobic cultures of the isolate MCF85) during culture incubation, as indicated by the equation:



It was not possible to ascertain whether MCF85 was able to grow by ferric iron respiration under anoxic conditions. Although cell numbers increased between day 0 and day 8, during this period there was limited reductive dissolution of schwertmannite, and the cell numbers declined sharply from day 8 onwards, probably due to attachment of the bacteria onto the mineral. A similar initial increase in cell numbers was observed in schwertmannite-free control cultures incubated anaerobically, but in this case the numbers remained stable until the end of the experiment. The initial increase in cell numbers in both control and schwertmannite-containing cultures was probably due to the presence of a small amount of dissolved oxygen in the culture medium. In contrast, cell numbers of MCF85 increased throughout incubation in cultures incubated under micro-aerobic conditions in the presence of schwertmannite, confirming its growth under such conditions.

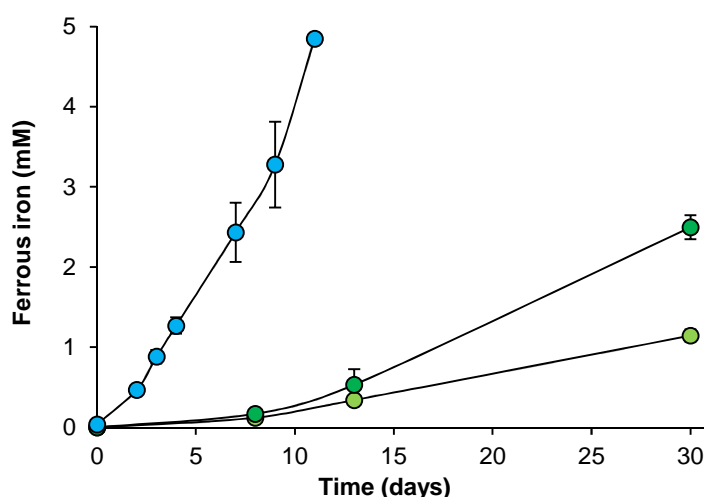


Figure 9. Reductive dissolution of schwertmannite by isolate MCF85 in cultures containing 100 mg of mineral incubated under micro-aerobic conditions (●) and in cultures incubated under anaerobic conditions containing either 50 mg (●) or 100 mg (●) of mineral.

4.3.4 SENSITIVITY TO TRANSITION METALS, ALUMINIUM AND SALT

The minimum metal concentrations that caused growth inhibition in the different isolates or minimum inhibitory concentrations are listed in Table 4.8. MCF85 isolate grew in media containing >100 mM of ferrous iron (Table 4.8), one of the most abundant metals in the Guadiana pit lake (Table 2.1 and 2.2). The majority of the isolates were

also very tolerant to aluminium, which is a very abundant in the lake waters (Table 2.2), except for isolate MCF105 that was less tolerant to this metal (Table 4.8). The isolates were also highly tolerant to some transition metals, though more sensitive to copper than to the other metals tested (Table 4.8). The isolates were also more tolerant to arsenic (V) (which occurs as anionic H_2AsO_4^- at the pH of the media used) than to As(III) (which occurs as undisassociated H_3AsO_3 in acidic solutions), except for MCF105, which was tolerant to similar concentrations of As(V) and As(III). Both reference acidophilic heterotrophic acidophiles (*Acidiphilium cryptum* SJH and *Ac. aromatica*^T) grew in the presence of 50 mM arsenic (V) and 1 mM arsenic (III).

The majority of the isolates had more similar tolerances to sodium chloride than to magnesium sulphate (Table 4.8). Isolate MCF85 grew in media supplemented with 500 mM (but not 700 mM) magnesium sulphate. However, its tolerance to sodium chloride was much less, with positive growth occurring in media supplemented with 100 mM NaCl, but inhibited by 200 mM NaCl.

Table 4.8. Minimum inhibitory concentrations (MIC) of some metal(oid)s and arsenic, with the highest concentrations at which the isolates grew indicated in parentheses. All the concentrations are mM.

	Minimum inhibitory concentrations (mM)					
	MCF9	MCF10	MCF14	MCF40	MCF85	MCF105
Al	250 (200)	(500) 300	250 (200)	100 (75)	200 (175)	5 (1)
As(III)	5 (1)	5 (1)	5 (1)	5 (1)	1.0 (0.5)	>10
As(V)	50 (10)	50 (10)	50 (10)	10 (5)	>50	>10
Cu(II)	1 (0.5)	1 (0.5)	1 (0.5)	1 (0.5)	10 (5)	0.05 (0.01)
Fe(II)	50 (25)	50 (25)	50 (25)	25 (10)	135*	50 (10)
Mn(II)	100 (50)	100 (50)	100 (50)	100 (50)	50 (10)	5 (1)
Ni(II)	50 (25)	50 (25)	50 (25)	75 (50)	50 (10)	5 (1)
Zn	25 (10)	25 (10)	25 (10)	100 (75)	75 (50)	50 (5)
MgSO₄	300 (200)	300 (200)	300 (200)	100 (50)	700 (500)	300 (200)
NaCl	300 (200)	200 (100)	300 (200)	300 (200)	200 (100)	300 (200)

* Largest concentration tested to avoid precipitation of ferrous phosphate.

In general, no clear evidence has been found of metal intracellular accumulation in any of the isolates. Isolate MCF85 was found to have extracellular deposits, which may

be caused by the accumulation of iron (Fig. 4.10). Although *Ac. aromatica*^T showed some intra-cellular accumulation, but it was not as clear as in MCF85 (Fig. 4.10).

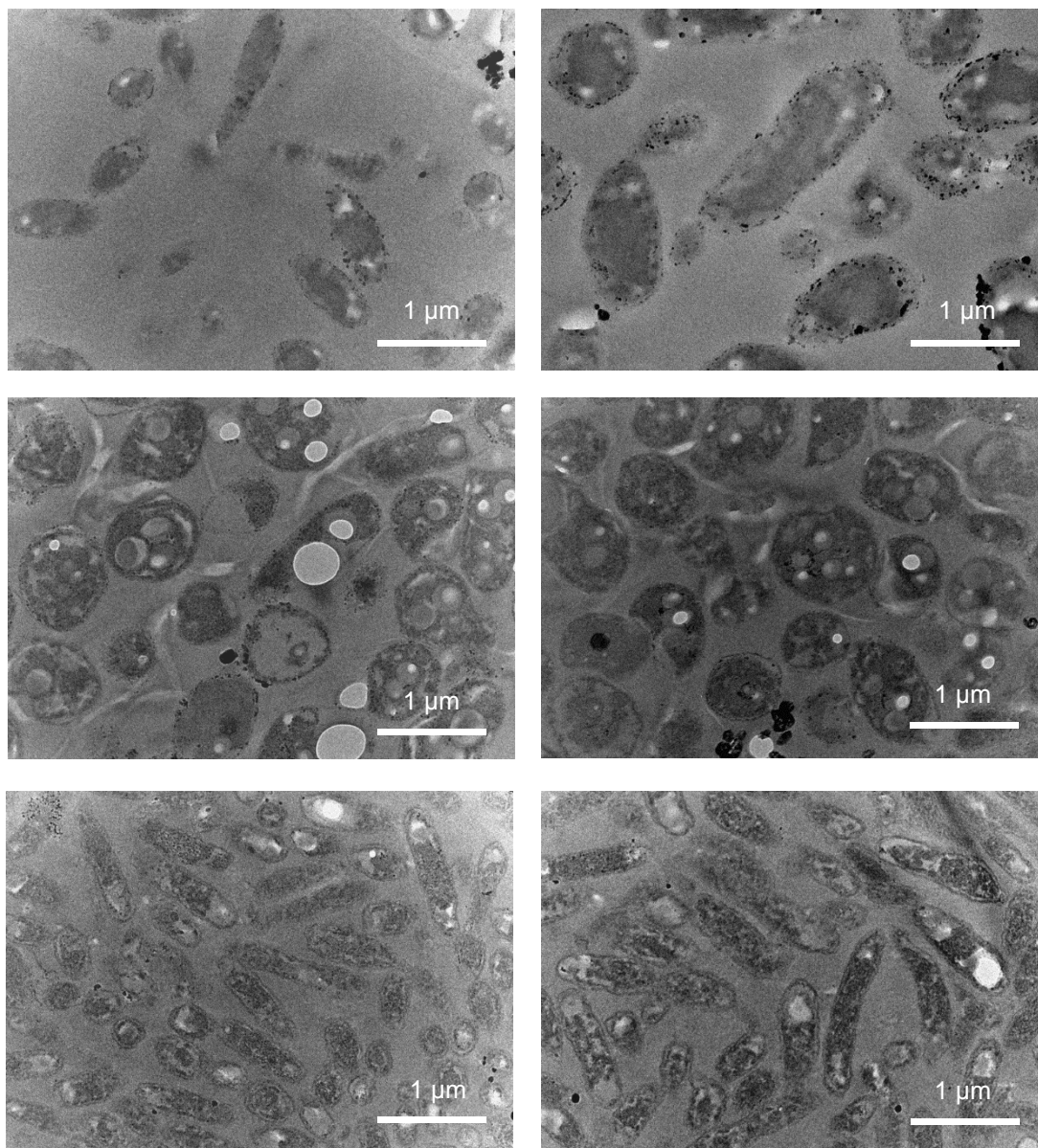


Figure 4.10. Transmission electron microscopy images of cell sections of some of the isolates growing in the presence of iron, MCF85 (top) showing extracellular deposits, and *Ac. aromatica*^T (middle), growing in the presence of aluminium, and MCF9 (bottom) growing in the presence of iron, showing vacuole-like structures.

The chemical analysis of these precipitates was unsuccessful as they were very small ($>0.1 \mu\text{m}$), although similar precipitates were found in the resin that could be identified as iron-rich precipitates. Some of the isolates appeared to develop a vacuole-

like structure in the presence of high metal concentration (Fig. 4.10) such as *Ac. aromatica*^T in the presence of aluminium or MCF9 in the presence of ferrous iron (Fig. 4.10). The formation of these structures is unknown. Fresh-prepared samples were surrounded by the growing liquid medium so that it is not clear if there is a cell-metal binding mechanism or just the remaining medium around the cells. Arsenate was not present in any of the samples.

4.4 DISCUSSION

Among the six isolates studied, four new species, and a new genus and species were found. Isolates MCF9, MCF10, MCF14 and MCF40 belong to the phylum *Acidobacteria*, MCF85 to the phylum *Proteobacteria* and MCF105 to the phylum *Firmicutes*.

4.4.1 ISOLATES MCF9, MCF10 AND MCF14

Isolates MCF9 and MCF14 are the same species, and both are acidophilic microorganisms. They present β -carotene as a pigment. Both isolates grew at pH between 3.2 and 5.2, and they can grow at 10°C but not at 40°C. The range of substrates that these isolates can use is similar; they are able to grow in a wide range of sugars including all the disaccharides, and there is a complete inhibition of growth in the presence of all the organic acids tested, and they cannot use aromatic compounds. Both isolates are able to grow in the presence of the same metal concentrations, both catalyse the reductive dissolution of ferric iron minerals such as schwertmannite under micro-aerobic conditions. Both acidophiles can grow under aerobic and micro-aerobic but not under anaerobic conditions.

Isolate MCF10 belongs to the same genus than isolates MCF9 and MCF14 and grows at 10°C but not at 40°C. MCF10 catalyses the reductive dissolution of ferric iron minerals such as schwertmannite under micro-aerobic conditions like the other two isolates. Moreover, MCF10 can grow under aerobic and micro-aerobic conditions but not under anaerobic conditions. Some other physiological features were found to be different indicating that this isolate can be classified as a different species than the other two. Isolate MCF10 is able to grow using lactic acid that causes growth inhibition in MCF9 and MCF14 and also is less tolerance to NaCl than the other two. In addition, it was estimated that this bacterium is able to grow at lower pH (between 2.3 and 5.1) than its

relatives, MCF9 and MCF14 (growth pH from 3.2 to 5.2), and does not produce pigments.

As both isolates MCF9 and MCF14 share the same physiological characteristics and differ with isolate MCF10, two new species are proposed, "*Acidicapsa ferrireducens*", the type strain is MCF9^T (= DSMZ 28997^T) and "*Acidicapsa acidophila*", the type strain is MCF10^T. Both strains were isolated from the Cueva de la Mora pit lake.

4.4.2 ISOLATE MCF40

The isolate MCF40 is able to grow at pH between 2.3 and 5.3, and grows at temperature of 10°C but not at 40°C, being classified as an acidophilic microorganism. This isolate is the only one among the aerobic heterotrophs studied that does not reduce iron, and only grows under aerobic conditions. This strain is not very tolerant to the presence of metals although is the most zinc tolerant within the studied isolates. Isolate MCF40 closest relatives belong to the genus *Granulicella* sp., any of the described species belonging to this genus grow at pH <3.0. Therefore, a new species "*Granulicella acidophila*" is proposed, the type strain is MCF40^T, which was isolated from the Guadiana pit lake (Herrerías mine, Spain).

4.4.3 ISOLATE MCF85

Isolate MCF85 is distantly related to any other characterized isolate and because of its novelty has been completely characterized. This isolate catalyses the reductive dissolution of ferric iron minerals such as schwertmannite under micro-aerobic and anaerobic conditions. It grows at 8-45°C (optimally at 32-35°C) and at pH 2.5-4.5 (optimally at 3.5), therefore is an acidophilic mesophile. A new genus *Acidibacter* gen. nov. and a new species *Acidibacter ferrireducens* ge. nov. are proposed. The type strain is MCF85^T (= DSMZ 27237^T = NCCB 100460^T), which was isolated from the Guadiana pit lake (Herrerías mine, Spain).

4.4.4 ISOLATE MCF105

The isolate MCF105 is only able to grow under strict anaerobic condition. The majority of the tested organic acids did not cause growth inhibition, but the aromatic compounds did. This isolate is an acid-tolerant mesophile, showing no growth at 10 nor

at 40°C, and growing on a large pH range (from 3.4 to 8.4). This isolate belongs to the genus *Clostridium* sp. The members of this genus are able to grow in large pH range but this is the first to grow at pH <3.5. This isolate is able to grow under arsenic (III) concentration over 10 mM unlike the rest of the other isolates. This aspect should be studied in the future as this species of arsenic is more toxic than As(V). A new species is therefore proposed: "*Clostridium acididurans*", the type strain is MCF105^T, which was isolated from a sediment sample obtained at 38 m in the Cueva de la Mora pit lake.

4.4.5 METAL TOLERANCE

Acid sulphate-rich environments originated after metal-mining activity present high concentration of dissolved metals (e.g. Nordstrom and Alpers 1999, López-Pamo et al. 2009, Rowe et al. 2007, Druschel et al. 2004, Kay et al. 2014). The knowledge of microbially mediated mechanisms of metal attenuation would provide an advantage for bioremediating and biomining activities. There are some studies about bacterial metal resistance (e.g. Nies 1999) including acidophiles (Dopson et al. 2003). Some of these studies have addressed the question of how metals affect bacterial morphology (Chakravarty and Benerjee 2008).

The majority of the isolates studied in this work were able to grow in the presence of high metal concentration as other acidophiles (Fischer et al. 2002, Hedrich and Johnson 2013b, Jones et al. 2013), although the anaerobic heterotroph isolate MCF105 was the least tolerant. This isolate has only been detected in sediments from Cueva de la Mora pit lake where the pH is higher (pH>4.5) and many of the metals are trapped in minerals under diverse forms, including oxy-hydroxysulphates (e.g. Al, hydrobsaluminite; Fe, schwertmannite, Sánchez-España et al. 2006, 2011, 2013) or as sulphides (Zn, Cu; Diez-Ercilla et al. 2014). No clear evidence of metal resistance mechanisms affecting bacterial morphology, intra- or extra-cellular accumulation of metals, has been found in any of the isolates studied. However, isolate MCF85 presented extracellular accumulation that could be attributed to iron precipitation, as similar iron precipitates were found within the resin in addition to that no evidences of these formations were found in the iron-free culture. An example of iron precipitation was found in the isolate *Acidiphilium* sp. PM, which was reported to induce the formation of Fe-carbonates in acid medium (Sánchez-Román et al. 2014). Whether isolate MCF85 is able to form similar iron precipitates to *Acidiphilium* sp. PM is still unknown, and further studies will be required. Another reported case of bacterially-induced metal (Al) precipitation was

mediated by sulphate-reducers (Meier et al. 2012). However such Al biomineralization has not been detected in any of the six heterotrophic bacteria studied.

Bacterial tolerance to metals has been shown to be significantly different among the isolates studied. All the aerobic isolates reported in this work were obtained from the upper layers of two metal-mine pit lakes, Cueva de la Mora and Guadiana. These lakes are characterized by relatively high metal concentrations (Table 2.1 and Table 2.2). Although diverse bacteria populate the upper and middle layers of both lakes, no bacteria was detected in the anoxic bottom layer (Chapter 3). The apparent lack of bacteria in these waters has been proposed to be related to the absence of suitable organic compounds for anaerobic heterotrophic bacteria such as sulphate-reducers (Chapter 3, Falagán et al. 2014). The data reported in this study show that metal toxicity is a major factor determining bacterial growth. In particular, copper and ferrous iron seem to be the most critical among all metals tested determining bacterial growth of the isolates studied. Ferrous iron nearly doubles the minimum inhibitory concentrations reported in Table 4.9 in the bottom layers of both lakes (Cueva de la Mora and Guadiana), and copper also exceeds these values in the upper forty meters in the Guadiana pit lake. And, although the majority of the isolates studied are aerobes, the results obtained during the metal tolerance tests suggest that the presence of dissolved metals in the lake waters would be another stress factor for bacterial growth, especially in the anoxic bottom layers.

4.5 CONCLUSIONS

One new genus and a new species of an acidophilic *Proteobacterium* have been described in this chapter. Moreover, other four new species of the genera *Acidicapsa* sp., *Granulicella* sp. and *Clostridium* sp. have been described. Further experiments will be carried out in order to finalize the complete characterization of these four new species.

All the isolates were obtained from acidic environments, in accordance with the finding that the majority of the studied isolates are acidophiles, and one of them is acid-tolerant (MCF105). The latter was found in sediments, where the pH is slightly higher than that of the water column where the others were obtained.

The isolates present different tolerances to metals. The most toxic metal for all of them was copper. The majority of the isolates were fairly tolerant to aluminium. Although there is not clear explanation for this, these two metals, copper and aluminium, are

present in very low and very high concentrations, respectively, in the water column of the lakes from which these isolates were obtained. Only one isolate (MCF105) obtained from sediments was more sensitive to the presence of dissolved metals. This is coherent since the sediment pore waters are known to have relatively higher pH (>4.5) in comparison to the overlying water.

Among all the metals tested, the tolerance to ferrous iron seems especially relevant since the concentration of this metal in the deepest part of the studied pit lakes is considerably higher than the minimum concentration required to inhibit growth of the isolates studied. This finding allows speculating that the unsuccessful detection of bacteria in the bottom layers of the two pit lakes could be related to the high metal concentration and the inhibitory effects that this ion has in bacterial growth.

No clear evidence of intra- or extra-cellular metal accumulation was observed in the isolates studied. Only one of the isolates (MCF85) presented extracellular precipitates (possibly iron precipitates). Further studies, such as genomic studies, will be required to determine the mechanisms by which these new species are able to resist the presence of metals.

4.6 APPENDIX

A.4.1. Fixation and embedding protocol for resin section TEM used in this work

1. Centrifuge the liquid culture at 15,000 g. Wash the pellet twice with sterile distilled water pH 1.7.
2. Fix samples with glutaraldehyde 2% in Sorensen buffer 0.1 M, pH 7.4. Leave the samples at least 1 – 4 hours at room temperature.
3. Wash three times for 10 – 30 minutes in phosphate buffer 4 -8 % sucrose.
4. Dehydrate the samples through a series of acetone:
 - i. 30% acetone 30 min
 - ii. 50% acetone 30 min
 - iii. 70% acetone 30 min
 - iv. 90% acetone 30 min
 - v. 100% acetone 30 min
5. Resin infiltration by embedding through a series of resin:
 - i. Acetone – epoxy resin (2:1) 60 min
 - ii. Acetone – epoxy resin (1:1) 60 min
 - iii. Acetone – epoxy resin (1:2) 60 min
6. Embed in 100% epoxy resin. The remaining acetone should evaporate.
7. Replace resin and place the fresh resin in embedding modules.
8. Leave 48 hours at 55°C to allow polymerization.

The resin-cell containing modules were cut at 70 nm.

**MICROBIAALLY MEDIATED
NITROGEN CYCLING IN
ACIDIC PIT LAKES OF THE
IBERIAN PYRITE BELT**

5

Results shown in this chapter have been included in a recent paper by Jeschke et al. (2013): No nitrification below pH 3.0. *Environ Sci Technol* 47: 14018-14023.

5.1 INTRODUCTION.....	128
5.2 MATERIALS AND METHODS	131
5.2.1 SAMPLING	131
5.2.2 DENITRIFICATION COUPLED TO IRON OXIDATION SET UP	131
5.2.3 NITRIFICATION EXPERIMENTS SET UP	133
5.2.4 LABORATORY-BASED CHEMICAL ANALYSES.....	134
5.3 RESULTS.....	134
5.3.1 DENITRIFICATION	135
5.3.2 NITRIFICATION	139
5.4 DISCUSSION.....	140
5.5 CONCLUSIONS.....	142

Abstract

Nitrogen is considered as a limiting nutrient that is found at low concentration in fresh waters. Acidic mine pit lake water chemistry has been studied widely in the last decades showing that the presence of inorganic nitrogen is mainly present as ammonium, although in low concentration. The primary production in two metal-mine pit lakes recently studied was limited by phosphorus availability. The distribution of nitrogen in the water column of these two lakes has been studied, ammonium concentration increases with depth, while nitrate is present at low concentration in the lake waters. The presence of bacterial denitrification (nitrate reduction coupled to iron oxidation) and nitrification in the lakes' water was determined. Several macrocosms were set under aerobic and anaerobic conditions. Nitrate, ammonium, dissolved organic carbon and iron were monitored during the experiments. Denitrification was studied in ferrous iron-containing samples under aerobic and anaerobic conditions. Nitrification was determined by adding the ^{15}N isotope into parallel macrocosms. Neither denitrification nor nitrification was detected in any of the samples. In some samples ferrous iron concentration increased along the experiment, which suggest the presence of iron reducers, although in other cases, ferrous iron concentration increased in the nitrate-free samples, which suggests that nitrate may be toxic for some iron reducers. The absence of nitrification would explain the accumulation of ammonium in the lake waters, although the origins of nitrate remain unanswered.

5.1 INTRODUCTION

Carbon, oxygen, nitrogen, phosphorus and sulphur are all nutrients needed in large quantities to enable growth of plants and animals, but these are usually scarce in lake waters (Goldman and Horne 1983). About 95% of a cell dry weight is composed by carbon, oxygen, hydrogen, nitrogen, sulphur, phosphorus, calcium, magnesium and iron. These elements are referred to as macronutrients as they are required in large quantities for cellular growth, while the oligoelements (e.g. Co, Cu, Fe, Mn, Si) are only necessary in small quantities. The first three macronutrients (C, O and H) are taken at once when assimilating organic molecules. The other macronutrients, nitrogen, phosphorus and sulphur, which are necessary to build organic molecules inside the cell (e.g. nitrogen for aminoacids, phosphorus for nucleic acids, sulphate for carbohydrates) are assimilated in different ways (e.g. incorporation of nitrate or ammonia in the case of nitrogen; oxidative phosphorylation in the case of phosphorus; utilization of cysteine and methionine in the case of sulphur) (Prescott et al. 2000). Because phosphorus and nitrogen are usually scarce in the environment, they are considered limiting nutrients (Prescott et al. 2000).

Phosphorus has been reported to be the main limiting nutrient for primary production in lakes in general (Wetzel 2011), and in acidic mining pit lakes (APL) in particular (Nixdorf and Kapfer 1998, Kleeberg 2013, Weithoff et al. 2013). As shown in Chapter 3, phosphorus also acts as a limiting factor for phytoplanktonic growth in the Cueva de la Mora and Guadiana pit lakes.

Rainfall, nitrogen fixation, runoff and groundwater are the main inputs of nitrogen in water. Losses of nitrogen may occur in the form of outflows from the water basin, coprecipitation with other compounds and reduction of nitrate to nitrogen gas, which may escape to the atmosphere (Wetzel 2001). Ammonia is generated by the biological dissimilation of nitrate, although it can also be a by-product of organic matter decomposition by heterotrophic bacteria, and can be produced by protists and flagellates that excrete ammonia (Wetzel 2001). The amount of nitrate present in water mostly depends on the surrounding environment of the water basin, changes in vegetation, land disturbance or farming, which may provoke an increase in its concentration (Goldman and Horne 1983). The distribution of nitrate and ammonium (ammonia in water is mainly present as ammonium, NH_4^+ ; Wetzel 2001) in freshwater lakes follow different tendencies depending on the trophic state of the lake (oligotrophic or eutrophic).

Nitrate is more abundant in the oxic layer of eutrophic lakes than in the anoxic bottom layer. Ammonium follows instead the opposite trend, being more abundant in the bottom anoxic layer. However, in oligotrophic lakes, nitrate is slightly less abundant in the upper layer than in the bottom layer and ammonium is at low concentration in the whole water column (Wetzel 2001). The majority of the acidic pit lakes reported in Friese et al. (2013) present values of $\text{N-NH}_4^+ < 1 \text{ mg/L}$ ($\text{NH}_4^+ < 0.07 \text{ mM}$), although the lignite and coal pit lakes in Germany present higher values up to 20 mg/L , N-NH_4^+ (1.4 mM NH_4^+) or $\sim 3 \text{ mg/L}$, NO_3^- (0.2 mM NO_3^-). Ammonium concentration increases as pH decreases in the lignite APLs of Germany, although no clear correlation has been found between pH and NO_3^- concentration. However, in some APLs of the Iberian Pyrite Belt (IPB), N-NH_4^+ concentration is higher in the anoxic bottom layer where the pH is > 3 ($2\text{-}24 \text{ }\mu\text{g/L N-NH}_4^+$ ($0.14\text{-}1.7 \text{ }\mu\text{M NH}_4^+$) in the upper layer vs. $41\text{-}336 \text{ }\mu\text{g/L N-NH}_4^+$ ($2.9\text{-}24 \text{ }\mu\text{M NH}_4^+$) in the bottom layer; Wendt-Potthoff et al. 2012, Sánchez-España et al. 2013, Santofimia et al. 2013).

Nitrogen is present as nitrate and ammonium in the water column of Cueva de la Mora and Gadiana pit lakes (Chapter 2), but the accumulation of these two elements has not been satisfactorily explained, and the microbial influence on the distribution and dynamics of this nutrient in the lake waters has been overlooked.

Microorganisms can incorporate nitrogen in different ways. Some are able to use atmospheric nitrogen (nitrogen fixation or reduction of N_2 to NH_4^+), while the majority of the microorganisms incorporate nitrogen as ammonium (ammonium uptake) or nitrate (nitrate assimilation). However, there are other microbiological processes where nitrogen is involved such as nitrification and denitrification (Prescott et al. 2000).

Nitrification is the aerobic oxidation of ammonium to nitrite (equation 1) which is further oxidized to nitrate (equation 2) during a second step. The first step is performed by microorganisms such as *Nitrosomonas* or *Nitrosococcus* while the second step is catalyzed by *Nitrobacter* or other chemolithotrophic bacteria. In addition, some heterotrophic bacteria and fungi are capable of performing nitrification in combination with denitrification under low oxygen concentration.



Although these processes represent the main path to oxidize ammonium to other oxidized forms of nitrogen, there are other routes also; e.g. *Nitrosopumilus* (which belong to the archaeal phylum *Thaumarchaeota*) is an ammonia-oxidizing *Archaea* (Madigan et al. 2015); *Nitrosomonas eutropha* is able to oxidize ammonium anaerobically to nitrite or nitric oxide (Prescott et al. 2000); and some heterotrophs (e.g. *Brocadia anammoxidans*) drive the anaerobic oxidation of ammonium to N₂ and N₂O in a process called Anammox (equation 3). Nitrite in equation (3) presumably comes from the aerobic ammonia-oxidizing *Bacteria* and *Archaea*, which coexist with the anammox bacteria (Madigan et al. 2014). Therefore, nitrification is not an exclusively aerobic process but also can occur under anoxic conditions (Prescott et al. 2000, Madigan et al. 2014).



Although nitrate can be reduced to nitrite, this process is not ideal as it requires too much of the first reactant and does not produce much energy. In addition, nitrite is toxic and its accumulation may cause severe consequences for the aquatic biota. In the dissimilatory nitrate reduction process, nitrate is reduced to NO, and only some microorganisms can reduce the first to ammonia (Madigan et al. 2014). Moreover, nitrate can be reduced to N₂ in a process called denitrification. The overall equation is:

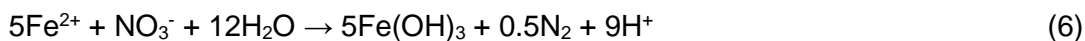


However, there are other intermediate steps in which other nitrogen species are involved:

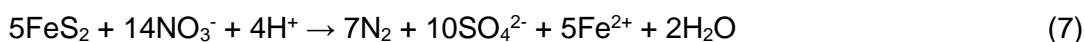


Although denitrification is an anaerobic process, it can take place under oxic conditions as an alternative to the aerobic respiration by some facultative anaerobes, e.g. *Pseudomonas* spp. and *Bacillus* spp. (Prescott et al. 2000).

Some microorganisms are able to couple nitrate reduction to iron oxidation, Carlson et al. (2013) proposes that all microorganisms able to perform nitrate reduction are innately capable of catalysing the oxidation of ferrous iron coupled to nitrate reduction. In this process, iron acts as an electron donor (equation 6).



In a similar equation, pyrite can act as an electron donor in anoxic groundwater environments (equation 7), (Jørgensen et al. 2009):



The aim of this study was to investigate the presence of a biogeochemical controlled nitrogen cycle in two acidic mine pit lakes of the Iberian Pyrite Belt, Cueva de la Mora and Guadiana, and to determine the microbial role in the distribution of nitrate and ammonium within the lake water columns. Given the low pH conditions and concentration of these two elements in both pit lakes, the conclusions emerging from this work could be valuable for the study of nitrogen cycling in other acidic lakes formed in abandoned mines with similar characteristics.

5.2 MATERIALS AND METHODS

5.2.1 SAMPLING

Aqueous samples were taken from the water column in two pit lakes, Cueva de la Mora and Guadiana, during a field campaign in September 2011. Samples were taken from different stratified zones in the two pit lakes using a 5 L Van Dorn sampler (KC-Denmark). Samples were obtained from Cueva de la Mora at 3, 8.5 and 35 m depths, and from Guadiana at 7, 20 and 50 m. In addition, a German acidic pit lake, Mining Lake 111 (ML111; 51°29' N, 13°38' E) was also sampled for comparison with the IPB lakes. Samples from ML111 were taken in September 2011 with a vertical sampling bottle at 0.5 m, 5 and 10 m. In all cases, the selected samples were chosen to represent shallow (oxic), transitional (chemocline) and deep (anoxic) conditions within the lakes.

Samples for determination of microbial nitrification and denitrification rates were stored at 4°C for a week until the experiments were performed. Water samples for chemical analyses (500-1000 mL) were filtered through sterile 0.2 µm (pore size) nitrocellulose membranes and N-free membranes. Filtered water was acidified with HCl and stored at 4°C until analysis was performed.

5.2.2 DENITRIFICATION COUPLED TO IRON OXIDATION SET UP

Water samples (triplicates) of the three pit lakes, Cueva de la Mora, Guadiana and ML111, were tested for denitrification in a short term experiment. Water samples (50 mL)

were placed into 100 mL serum bottles (macrocosms, Fig. 5.1) and cultivated for 25 days. Ferrous iron was added to each bottle to reach a final concentration up to 8 mM (water samples from the mixolimnion did not contain any ferrous iron whilst those from the monimolimnion contained ~3 mM ferrous iron). 5 mM of NO_3^- was added to a set of flasks (in the future referred to as “nitrate samples”) and a control was established by adding distilled water into another set of flasks (in future referred to as “control samples”). Anoxic conditions were created by fluxing N_2 into the sample-containing serum bottles. Macrocosms were incubated in the dark at 30°C. Nitrate, ammonium and iron speciation were measured in the course of the experiment (every 5-7 days), while pH, and dissolved organic carbon (DOC) were measured at the beginning and at the end of the experiment.

A parallel long-term denitrification experiment was carried out with the same samples. The same parameters measured in the short-term experiment were monitored, although in this case the incubation was under strictly anaerobic conditions and lasted 131 days. Sampling took place at the beginning and at the end of the long-term experiment.

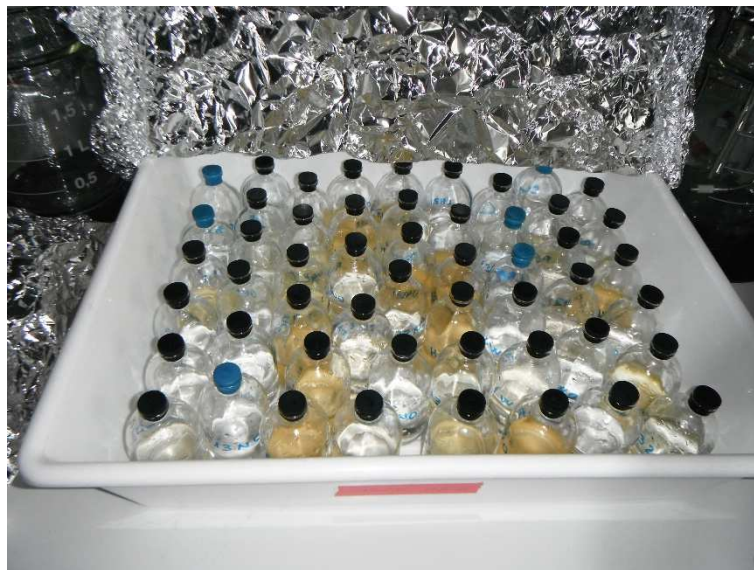


Figure 5.1. Serum bottles utilized during the short-term denitrifying experiment (macrocosms). The yellowish coloration of some of the macrocosms was caused by ferric iron precipitates accumulating on the bottle inner surface, as a response to the exposure to oxygen during the transportation of the water samples.

5.2.3 NITRIFICATION EXPERIMENTS SET UP

Water samples (triplicates) of the three lakes (Cueva de la Mora, Guadiana and ML111) were also tested for nitrification through stable isotope analyses. The samples were pre-incubated and shaken under aerobic and anaerobic conditions overnight. Then, $^{15}\text{NH}_4\text{Cl}$ was added up to 10% of the NH_4^+ concentration in the original water sample (Fig. 5.2). When samples contained low concentration of nitrate, 5 mM final concentration of NO_3^- (as NaNO_3) was added to each sample. Control was made by adding ATU (allylthiourea) as it is an inhibitor of nitrification (Hall 1984, Roy and Knowles 1995). The macrocosms were only sampled twice, at the beginning and at the end of the experiment (Jeschke et al. 2013). After the incubation period (24 hours), diluted (1:20), filtered and sterilized (0.2 μm pore size aceto-celulose membrane filters) samples were inoculated with a known denitrifying bacteria, *Pseudomonas chlororaphis* (ATCC 13985^T), which uses NO_x^- to form N_2O , in serum bottles under anaerobic conditions. Anaerobic conditions were created by bubbling the filter-sterilized medium with helium. This denitrifying bacterial activity was interrupted by the addition of NaOH to the bacterial culture. $^{15}\text{N}_2\text{O}$ was measured in 100 mL of gas sample taken from the inside of the macrocosms.



Figure 5.2. Conical flasks (aerobic) and serum bottles (anaerobic) utilized during the nitrifying experiment. The yellowish coloration of some of the macrocosms is caused by ferric iron precipitates formed in the monimolimnetic samples when exposed to air.

5.2.4 LABORATORY-BASED CHEMICAL ANALYSES

Analysis of ammonium- and nitrate-nitrogen and phosphate from water samples were carried out on site and around 2-6 hours after sampling. Water analyses were carried out with a portable UV-VIS DR2800 spectrophotometer (Hach) and Lange cuvette tests. The methods used were LCK 349 (0.05-1.50 mg/L P- PO_4^{3-} ; 0.005-0.147 mM PO_4^{3-}), LCK 385 (3-30 mg/L, dissolved organic carbon, (DOC; 0.25-2.5 mM DOC), LCK 304 (0.015-2.0 mg/L N- NH_4^+ ; 0.001-0.142 mM NH_4^+). The methods LCK 349 and LCK 358 required a previous digestion with a Hach-Lange LT100 thermostat, and in the case of the LCK 385, samples were shaken for 5 minutes with a Hach-Lange TOC-X5 shaker.

Nitrate was measured with an ion-selective electrode Nitrate ionplus[®] Sure-flow[®] and reference electrode 9307BNWP coupled to an Orion StartTM meter (Thermo Fisher Scientific Inc.). Calibration was performed with different standards prepared from a NO_3^{2-} standard (1,000 mg/L) supplied by the company.

Analyses of the samples from the denitrification and nitrification experiments were carried out at the UFZ facilities at Magdeburg before and after the experiments. Iron speciation was determined by the ferrozine assay (Stookey 1970). $^{15}\text{N}_2\text{O}$ was analysed with a mass spectrophotometer at the UFZ-Halle facilities with GC-MC. DOC, NO_3^- and NH_4^+ were measured at the UFZ-Magdeburg facilities. Nitrate and ammonium were analysed photometrically by continuous-flow analysis. DOC was analysed by infrared spectrometry with a TOC-analyzer (Dimatec).

Nitrification rates were calculated from the initial ($^{15}\text{NO}_x^-_{(i)}$) and final ($^{15}\text{NO}_x^-_{(f)}$) $\delta^{15}\text{N}$ of dissolved nitrate, nitrate concentration ($\text{NO}_x^-_{(f)}$), the quantity of added isotope tracer ($\alpha = ^{15}\text{N}$ content of NH_4^+), and incubation time. The isotopic analyses were converted to per thousand. The detection limit for $^{15}\text{NO}_x^-$ was 0.4 ‰ and, therefore, 100 nM/d (Jeschke et al. 2013).

5.3 RESULTS

Nutrient concentration in the lake waters were found to be higher in the monimolimnion than in the upper part of the water columns (Fig. 5.3), with the exception of nitrate which was lower in the bottom layer of the Cueva de la Mora pit lake. The oxic

layers and the transitional layers of both lakes are characterized by very low concentration of ammonium (1.1-5.3 μM) and phosphate (<6 μM), whereas in the bottom anoxic layer these two nutrients are more abundant (23-48 μM ammonium, 8-690 μM phosphate).

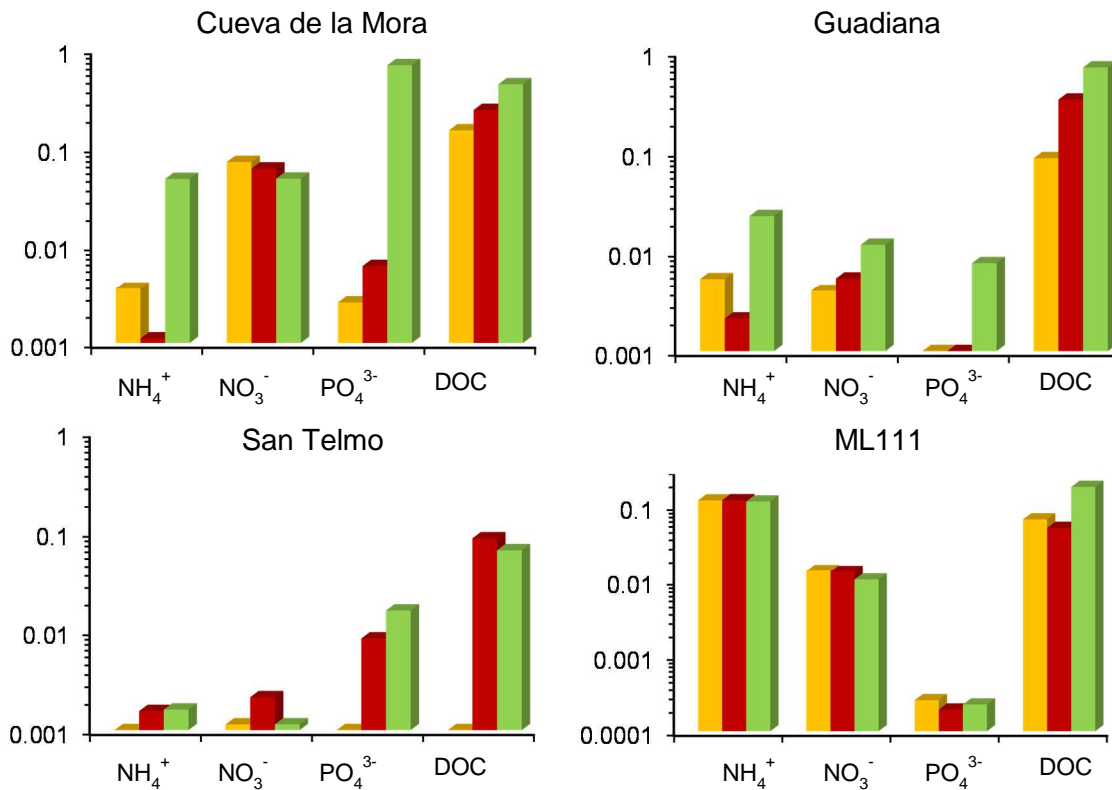


Figure 5.3. Nutrient concentrations (mM) in the water columns of the metal mine pit lakes Cueva de la Mora, Guadiana and San Telmo (Iberian Pyrite Belt) and in the coal-mine lake 111 (ML111; Germany). ■, oxic layer; ■, transition layer; ■, anoxic layer.

5.3.1 DENITRIFICATION

No changes on ammonium concentration were detected in the long-term experiment. With just a few exceptions, the DOC concentration increased during the experiment, which may have been caused by an increase in microbial biomass, autolysis or secretion of organic compounds (Table 5.1). In Cueva de la Mora samples, nitrate concentrations were slightly higher at the end of the experiment, and lower in the ML111 samples. The nitrate increase could have been caused by the release of senescent cells and subsequent autolysis, where DOC also increased. Moreover, nitrate and DOC increases could have been caused by the reduction of ferric iron compounds by which these two solutes could have been adsorbed. The decrease of nitrate in ML111 samples

may be explained by microbial nitrate fixation. Only three samples showed evidences of iron reduction (Table 5.1).

Table 5.1. Changes in pH, ferrous iron, nitrate, ammonium and dissolved organic carbon concentrations during the long-term (131 days) experiment. Numbers in bold indicate samples that experienced significant concentration changes. CM, Cueva de la Mora; G, Guadiana; DOC, dissolved organic carbon.

	Depth	pH		Ferrous iron	
	m	Start	End	mM	mM
				Start	End
Cueva de la Mora	3	2.59	2.61±0.02	3.83±0.18	3.79±0.15
	8.5	2.63	2.69±0.03	3.84±0.14	3.71±0.05
	35	3.62	3.44	53.27±4.28	51.18±2.6
Guadiana	7	2.97	3.22±0.08	3.84±0.28	4.04±0.21
	20	2.91	2.77±0.01	0.81±0.06	2.30±0.1
	50	4.19	3.68±0.25	57.30±2.48	57.56±1.23
ML111	0	2.62	2.55±0.01	3.81±0.2	4.62±0.1
	5	2.67	2.56±0.01	3.69±0.07	4.95±0.07
	10	3.02	2.94±0.03	2.56±0.04	2.52±0.8

Table 5.1. Continued.

	Depth	NO ₃ ⁻		NH ₄ ⁺		DOC	
	m	mM	mM	mM	mM	mM	mM
		Start	End	Start	End	Start	End
Cueva de la Mora	3	4.75	5.64±0.2	0.01	0.00	0.043	0.1±0.05
	8.5	6.13	6.12±0.6	0.01	< 0,003	0.12	0.14±0.002
	35	5.58	6.52±0.17	0.26	0.27	0.21	0.32±0.002
Guadiana	7	5.92	6.45±1.08	0.02	0.00	0.075	0.24±0.04
	20	5.84	5.84±1.01	0.03	0.00	0.07	0.15±0.001
	50	4.65	4.46±0.62	0.24	0.24±0.01	0.54	0.51±0.05
ML111	0	5.62	4.46±0.4	0.15	0.15	0.11	0.18±0.01
	5	5.05	4.82±0.21	0.15	0.15	0.21	0.41±0.04
	10	5.35	4.41±0.4	0.23	0.23	0.5	0.67±0.02

During the short-term experiment, no evidence of nitrate consumption or production was detected in the nitrate samples (Fig. 5.4) nor in the control samples. Only some DOC and iron concentration showed some changes (Table 5.2, Fig. 5.5). Ferrous iron concentration decreased in the Guadiana 20 m samples (nitrate and control samples),

which may have been caused by microbial iron oxidation or deposition of ferric iron compounds; in the same samples DOC was slightly higher at the end of the experiment. The DOC concentration in the control samples showed more significant changes than those of the nitrate samples, and the DOC content increased in the monimolimnetic samples of Guadiana and Cueva de la Mora whilst decreased in the others (Table 5.2).

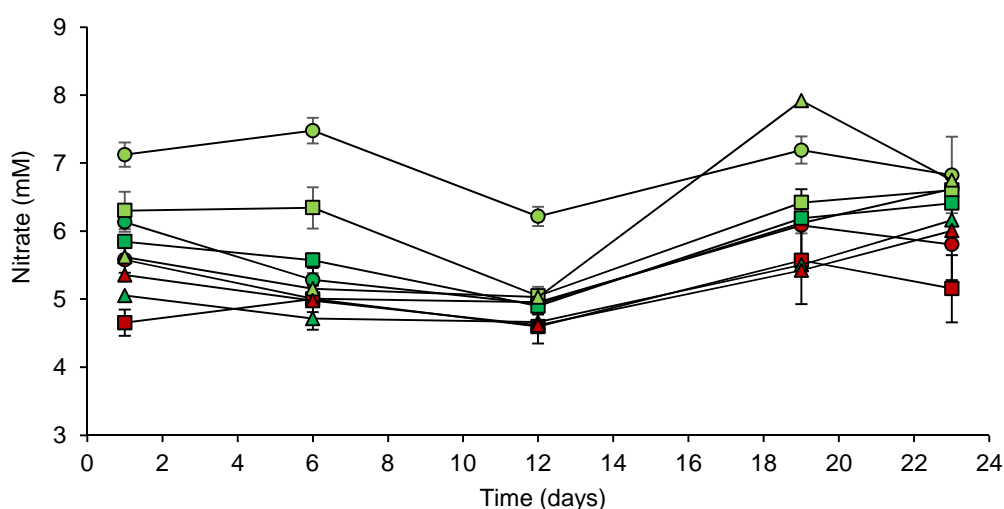


Figure 5.4. Evolution of nitrate concentration in the nitrate samples from Cueva de la Mora (CM), Guadiana (G) and ML111 during the short-term denitrification experiment. ●, CM 3m; ●, CM 8.5; ●, CM 35m; ■, G 7m; ■, G 20m; ■, G 50m; ▲, ML111 0m; ▲, ML111 5m; ▲, ML111 10m.

Table 5.2. Dissolved organic carbon (DOC) and ammonium (NH_4^+) concentration at the beginning and at the end of the short-term denitrification experiment. All concentrations are in mM.

		Nitrate samples				Control samples			
		DOC		NH_4^+		DOC		NH_4^+	
		Start	End	Start	End	Start	End	Start	End
Cueva de la Mora	3	0.04	0.05±0.04	0.01	0.01	0.04	<	0.01	0.01
	8.5	0.12	0.12±0.07	0.01	0.01	0.12	<	0.01	0.01
	35	0.21	0.21±0.23	0.26	0.26	0.21	1.13±0.04	0.26	0.26
Guadiana	7	0.08	0.08±0.09	0.01	0.01	n.a	n.a	n.a	n.a
	20	0.07	0.07±0.09	0.03	0.01	0.07	0.06±0.03	0.01	0.01
	50	0.54	0.54±0.44	0.24	0.24	0.54	1.13	0.03	0.24
ML111	0	0.11	0.11±0.08	0.15	0.15	0.11	<	0.24	0.15
	5	0.21	0.21±0.22	0.15	0.14	0.21	0.15±0.03	0.15	0.14
	10	0.48	0.48±0.56	0.23	0.23	0.48	0.31±0.05	0.23	0.23

<, below detection limit.

n.a., not analysed.

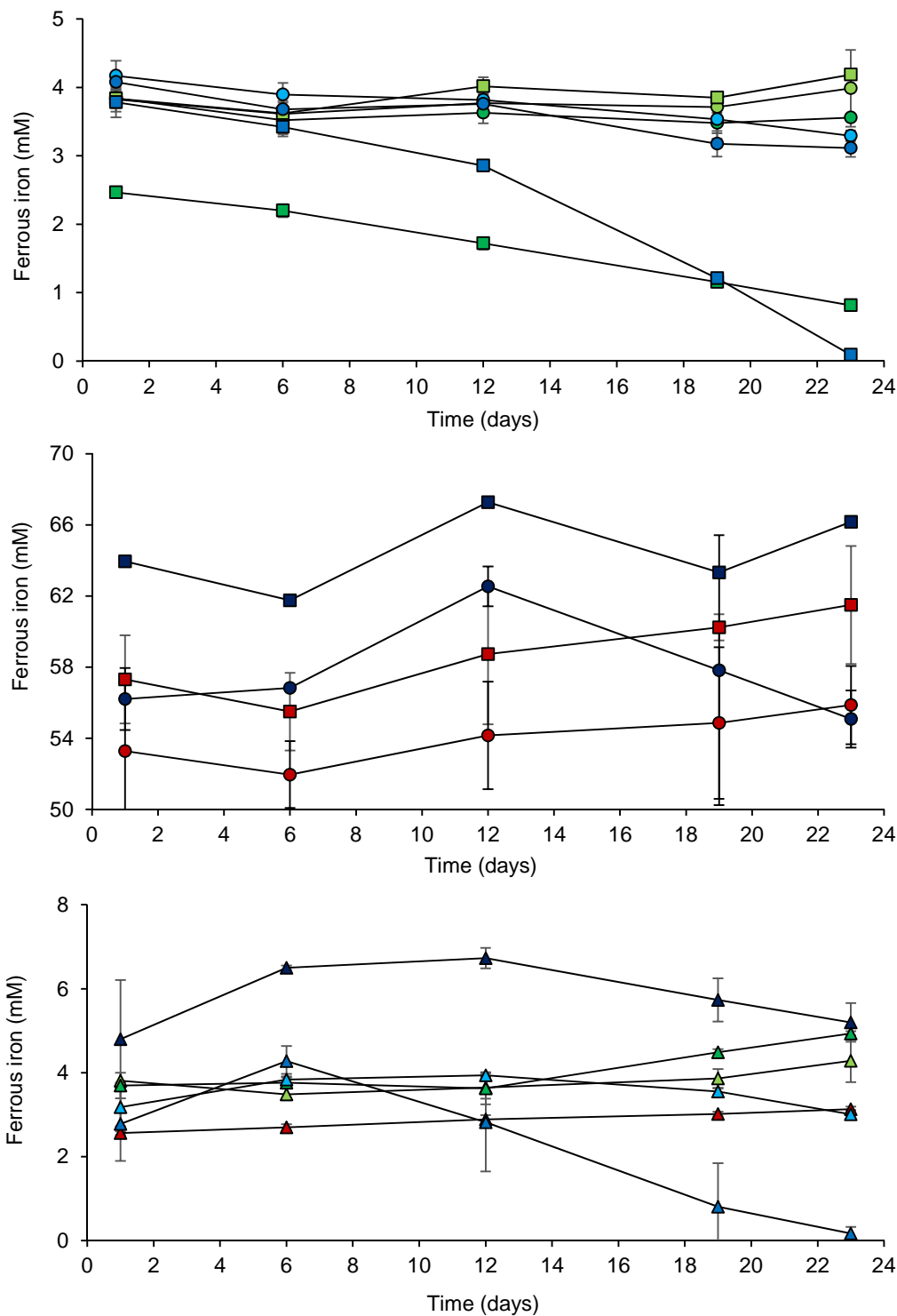


Figure 5.5. Ferrous iron concentration of the nitrate and control samples of Cueva de la Mora (CM), Guadiana (G) (middle) and ML111 (bottom) during the short-term denitrification experiment. Nitrate samples: ●, CM 3m; ●, CM 8.5; ●, CM 35m; ■, G 7m; ■, G 20m; ■, G 50m; ▲, ML111 0m; ▲, ML111 5m; ▲, ML111 10m. Control samples: ●, CM 3m; ●, CM 8.5; ●, CM 35m; ■, G 7m; ■, G 20m; ■, G 50m; ▲, ML111 0m; ▲, ML111 5m; ▲, ML111 10m.

5.3.2 NITRIFICATION

No significant changes in ferrous iron, nitrate or ammonium concentration were detected in any of the samples during the duration of the experiment (Fig. 5.6). The isotope ^{15}N concentration was below the detection limit in the two phases of the experiment (when macrocosms with APL non-sterilized water were incubated with ^{15}N , and when the filtered sterilised ^{15}N incubated APL water samples were incubated with an inoculum of the denitrifying bacteria *Pseudomonas chloroaphis*). Therefore, the nitrification ratio was below the detection limit, which indicates the absence of nitrification in the water samples.

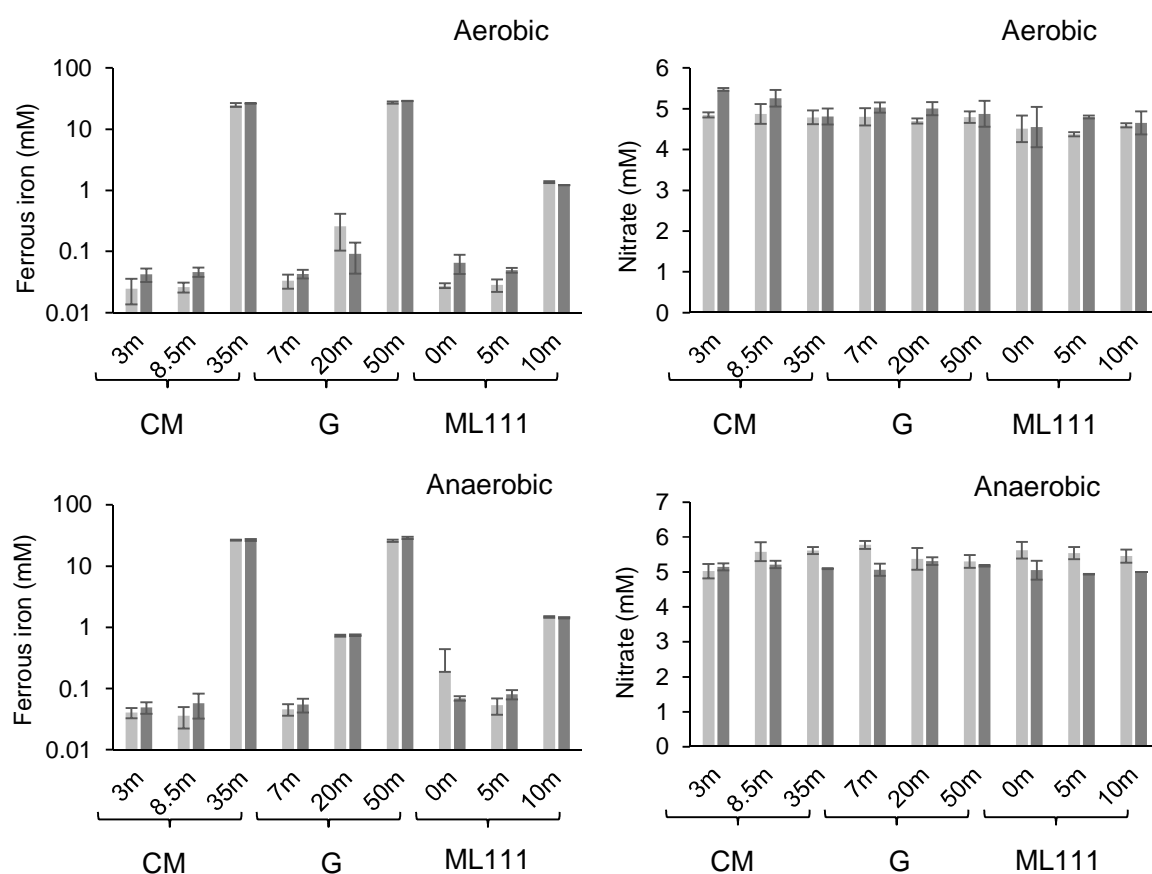


Figure 5.6. Ferrous iron and iron concentration after 24 h incubation (nitrification experiment) under aerobic (top) and anaerobic conditions (bottom). Light grey bars, beginning of the experiment; dark grey bars, end of the experiment. CM, Cueva de la Mora; G, Guadiana; ML111, Mining lake 111.

5.4 DISCUSSION

Nutrients availability strongly influences the composition and structure of the microbial community of aquatic systems (Ehrlich 2000, Wetzel 2001, Konhauser 2006). Phosphorus is a key limiting nutrient in acidic mine pit lakes, as it is usually scarce and determines the growth of microorganisms. In Cueva de la Mora phosphorus is very scarce in the upper mixolimnion (Table 2.1), whilst more abundant in the lower monimolimnion of Cueva de la Mora and Guadiana pit lakes. This element distribution seems to affect the development of the microbial community making the transitional layer the most suitable environment to be inhabited. Phosphorus is bound to ferric iron in the upper layer while in the anoxic bottom layer (containing only ferrous iron) is more readily available as an inorganic form (orthophosphate, PO_4^{3-}). In the chemocline, both particulate (e.g. phosphates of iron (III) and other metals, adsorbed phosphate to ferric particles) and dissolved phosphate forms may coexist. Therefore, this transition layer presents the appropriate geochemical conditions for the development of a microbial community as shown in Chapter 3.

The results presented in this study show that there is no denitrification or ferrous iron oxidation coupled to nitrate reduction in the water column of the pit lakes studied. This observation is in agreement with previous studies, although the majority of the research has been performed at $\text{pH} > 5$, demonstrating that the denitrification process is affected by pH (Simek and Cooper 2002, Pan et al. 2012). For example, Saleh-Lakha et al. (2009) showed that the denitrification gene was negatively affected at $\text{pH} 5$. Glass and Silverstein (1998) determined that denitrification is inhibited at low pH . Burford and Bremner et al. (1975) state that the reduction of N_2O to N_2 is strongly inhibited at $\text{pH} < 7$. In other works, some authors claim that the optimum pH for denitrification is between 5.8-6 to 8-9.2 (e.g. Tang et al. 2010). Moreover, at $\text{pH} > 3.4$ nitrite is present as an anion, but at $\text{pH} < 3.4$ it is undissociated as a weak acid (HNO_2 , pK_a 3.4). The accumulation of HNO_2 inhibits the denitrification process (Glass et al. 1997).

The redox couples involved in the denitrification process have different redox potentials (E°) for the $\text{NO}_3^-/\text{NO}_2^-$ couple ($E^\circ = +350$ mV), and for the $\text{NO}_3^-/0.5\text{N}_2$ couple ($E^\circ = +710$ mV) (Hedrich et al. 2011). The bacterial use of electropositive couples is more favourable than those of less electropositive potentials (at low pH the redox potentials of the $\text{O}_2/\text{H}_2\text{O}$ couple is +1.12 V at $\text{pH} 2$ and that of the ferrous/ferric iron couple is +770 mV) (Hedrich et al. 2011). The NO_3^-/N_2 couple redox potential is similar to that of the iron

couple, thus both redox pairs may potentially be used. Some reasons to explain that nitrate is not used as an electron acceptor may be that both oxygen and iron are more available than nitrate in acidic environments, in addition to nitrite (intermediate of the denitrification process, equation (5)) being very toxic for microorganisms when present as nitrous acid at low pH. Therefore, the possibility that denitrification could take place in low pH environments is very low, if at all possible.

The slight changes of pH and ferrous iron detected in the samples during the experiments might have been caused by the precipitation of iron-hydroxydes (as evidenced by the formation of orange-brownish precipitates on the flask walls), which is a proton-releasing process. On the other hand, pH variation could be caused by iron oxidation or iron reduction. Although some small variations were observed in the ferrous iron concentrations during the denitrification experiments, these changes are not very marked. Many heterotrophs inhabiting these environments have shown their capacity to reduce iron (Johnson and Bridge 2002, Falagán and Johnson 2014 and Chapter 4), so their metabolisms could have caused such changes after the incubation period. In the ML111 samples, during the denitrification short-term experiment the control samples showed a significant reduction of ferrous iron. Nitrate can have poisoning effects on bacteria and, therefore, some microbial species would have suffered this effect and inhibit their activity, which is also reflected in the DOC concentration (Table 5.2).

Nitrification has not been described at pH <3.7. Jeschke et al., (2013) shows that there is no nitrification at pH <3. A study was completed using different water samples from some acid pit lakes (including data shown in this chapter from the Cueva de la Mora and Guadiana pit lakes water samples) to detect nitrification. Nitrification was found in none of these samples, not even in sediment samples. It is proposed that nitrification might be inhibited by high proton concentration or high metal concentration. And, at acid pH ammonium can be accumulated due to the absence of nitrification. However, a sediment sample of an acidic mine pit lake was found to be enriched on $^{15}\text{NO}_x$ (control samples too). The authors proposed that ammonium oxidation might be performed by archaea that are less sensitive to ATU (Lehtovirta-Morley et al. 2013). Clones from the new archaeal group *Thaumarchaeota* have been found in sediments of Cueva de la Mora pit lake (Falagán et al. 2014); the few validated species belonging to this group are chemolitho-autotrophic ammonium oxidizers (Pester et al. 2011), one member of this group, *Nitrosotalea devanaterrea*, has been isolated from acidic agricultural soils with pH 4-5 (Lehtovirta-Morley et al. 2011). Although no evidence of ammonium oxidation was

found in water samples of the acidic pit lakes, the presence of the archaeal clones in sediments, possibly ammonium oxidizing archaea, and the detection of $^{15}\text{NO}_x$ in Jeschke et al. (2013) research leaves open the possibility that ammonium oxidizing activity might occur in sediments in these acidic environments. Although, bacterial nitrification in water may be inhibited at $\text{pH} < 3$ as shown in this work and in Jeschke et al. (2013), not much is known about this new group of ammonium oxidizing archaea. Moreover, sediments in acidic pit lakes present $\text{pH} > 3$ (Chapter 2), more investigation to understand whether the two processes studied in this chapter could take place in these extreme environments should be performed in the future.

Inorganic nitrogen is present as nitrate and ammonium in the lakes. Nitrate has only been detected in the mixolimnion and the chemocline, while ammonium only presents significant and measurable amounts in the anoxic water of the monimolimnion (Fig. 5.3). The origin of nitrate in the lake waters is still uncertain. No nitrification has been detected in the lake waters, which brings into question where the nitrate comes from. The sources of nitrate are diverse, as this nutrient is quickly washed out from the soil. If the surrounding environment has suffered any kind of disturbance such as farming, fires may contribute to increase the amount of nitrate in the lake waters, in addition to that accumulated through precipitation. There are many environmental factors contributing to the final concentration of inorganic nitrogen in the studied acidic pit lakes, but there may also be a microbial contribution to the final bulk of nitrate and ammonium that has been overlooked.

5.5 CONCLUSIONS

Denitrification has not been detected in the water column of the acid pit lakes. The slight changes on nitrate during these experiments are not significant. This agrees with the lack of literature about denitrification at $\text{pH} < 5$. Denitrification coupled to iron oxidation is absent in the water column of these extreme environments.

Nitrification was not detected in any of the studied samples. As Jeschke et al. (2013), demonstrates, this process does not take place at $\text{pH} < 3$, which causes ammonium to accumulate in the waters of the acid mine lakes. The pH limit for nitrification should be between 3.0 and 6.8 as no other pH was tested during the experiments. Therefore, further experiments would be necessary to determine whether nitrification is possible at that range of pH. The presence of nitrate in the mixolimnion of the lakes might be caused

by the phytoplankton activity, as cells assimilate nitrogen during growth and release it when the cells die. Moreover, the runoff may contribute to the nitrate bulk of the lakes.

On the contrary, the detection of possibly ammonium-oxidizing archaea by Falagán et al. (2014) in the lake sediments provides evidence that the microbial composition in sediments of pit lakes is complex. Further research is required to determine whether ammonium oxidation or nitrate reduction take place in these acidic environments; it is not feasible in the water column, it may be in the lake sediments

**BIOLOGICALLY-INDUCED
PRECIPITATION OF
ALUMINIUM IN SYNTHETIC
ACID MINE WATER**

6

6.1 INTRODUCTION.....	148
6.2 MATERIALS AND METHODS	151
6.2.1 BIOREACTOR SETTINGS.....	151
6.2.2 ANALYTICAL METHODS.....	154
6.2.3 BIOMOLECULAR ANALYSES.....	155
6.3 RESULTS.....	156
6.3.1 BIOREACTOR.....	156
6.3.2 ANALYSIS OF PRECIPITATES.....	158
6.3.3 BACTERIAL BIOREACTOR POPULATION	160
6.4 DISCUSSION.....	162
6.5 CONCLUSIONS.....	166

Abstract

There is no known biological function associated with aluminium, and it is toxic for many organisms when bio-available (soluble) in high concentrations. Aluminium forms Al-hydroxides in fresh water at high pH, whereas at pH <2.0 it is present as the free cation, Al³⁺. Acid mine water is rich in sulphate which complexes with aluminium to form oxy-hydroxysulphates as felsőbányaite (also known as basaluminite) or hydrobasaluminite. An anaerobic bench-scale reactor system populated by a mixed community of acidophilic sulphate-reducing bacteria was tested to grow in the presence of elevated aluminium concentrations. The sulphate-reducing bacteria utilized protons released when soluble aluminium hydrolysed to form oxy-hydroxysulphates in the reactor, together with those present in the acidic (pH 3.0) feed liquor, to reduce sulphate, coupling this with the oxidation of glycerol. The two aluminium hydroxysulphates formed into the bioreactor were identified as hydrobasaluminite and felsőbányaite. The bacterial community was shown to reduce in diversity when the bioreactor was exposed to aluminium. The bioreactor population was mainly dominated by the sulphate-reducer *Desulfosporosinus acididurans* and, in a lower proportion, *Acidocella aromatica* and *Acidithiobacillus ferrooxidans*. This study aimed to test the hypothesis that it is possible to remove aluminium from contaminated acid waters via biosulphidogenesis.

6.1 INTRODUCTION

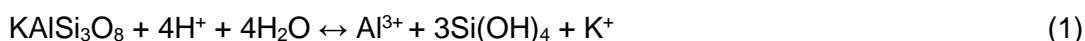
Aluminium is the most abundant metal of the continental crust, comprising the 8.3% (by mass) of the lithosphere (Turekian and Wedepohl 1961, Taylor 1964). This metal occurs primarily as aluminosilicates, although the major aluminium ore is bauxite, which consist mainly of Al-hydroxides. The unique aluminium oxidation state is +3. Aluminium is a metal with no known metabolic role and is generally toxic to bacteria, higher plants, humans and aquatic organisms (Macdonald and Martin 1988, Gensemer and Playle 1999, Krewski et al. 2007). This element is toxic to fish causing both respiratory and ion-regulatory effects (Gensemer and Playle 1999). Different algal species reduce their activity by ~50% in the presence of aluminium, which affects the photosynthesis or nutrients uptake, mainly below pH ~6.0 (Gensemer and Playle 1999). Aluminium is also toxic for many prokaryotic microorganisms. Bacterial species display different degrees of aluminium tolerance, e.g. *Acidocella aluminidurans*, 500 mM (Kimoto et al. 2010), *Rhodrorula glutinis*, 100 mM (Kawai et al. 2000), *Acidibacter ferrireducens*, 200 mM, (Falagán and Johnson 2014), *Escherichia coli* growth rate decreases as aluminium concentration increases in the growth medium (Bojic et al. 2002).

Aluminium is a metal present in acidic mine water, often in relatively high concentrations. Aluminium speciation in fresh water has been studied in depth (Ball and Norstrom 1989, Stumm and Morgan 1996) and is dominated by Al-OH species (Macdonald and Martin 1988, Gensemer and Playle 1999) whereas, in acid mine drainage (AMD) environments is dominated by Al-hydroxysulphates (Sánchez-España 2007). Aluminium speciation in AMD environments is strongly determined by the activity of the sulphate ion (SO_4^{2-}), (Nordstrom and Alpers 1999, Bigham and Nordstrom 2000, Sánchez-España 2007). At pH 2.5-3.5 and in the presence of sulphate (0.1 M), aluminium is present as sulphate complexes such as AlSO_4^+ and $\text{Al}(\text{SO}_4)_2^-$, and a minor presence of this element as the free cation (Al^{3+}). However, aluminium speciation and the activity of the sulphate anion both vary with pH. Only the free cation (Al^{3+}) is dominant at very low pH (<1.0), and sulphate complexes are dominant at pH 1.5 to 6.0 (Sánchez-España 2007). Within this pH range, Al sulphates and hydroxysulphates are formed as a result of Al hydrolysis and precipitation (Table 6.1). Beyond pH 6.0, aluminium sulphate complexes are replaced by aluminium hydroxide forms ($\text{Al}(\text{OH})_2^+$, $\text{Al}(\text{OH})_3$) (Sánchez-España 2007).

Table 6.1. Aluminium phases/minerals precipitating in AMD as a function of pH (Modified from Sánchez-España 2007).

Mineral	Formula	pH range
Gibbsite	Al(OH) ₃	>4.5-5.0
Alunite	KAl ₃ (SO ₄) ₂ (OH) ₆	3.5-5.5
Jurbanite	Al(SO ₄)(OH) · 5H ₂ O	<4.0
Felsöbányaite	Al ₄ (SO ₄)(OH) ₁₀ · 4H ₂ O	>4.5-5.0
Hydrobasalumnite	Al ₄ (SO ₄)(OH) ₁₀ · 12-36H ₂ O	>4.5-5.0

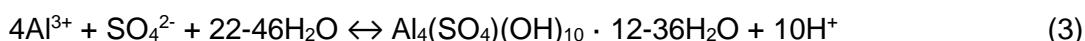
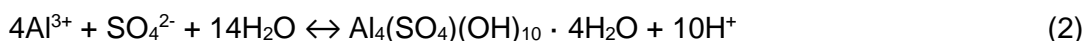
The main source of aluminium in these extreme environments is the weathering of aluminosilicates that are susceptible to proton attack (equation 1), a process that can be indirectly mediated by bacteria. For example, this process is enhanced during microbially-mediated pyrite oxidation by the generation of sulphuric acid (Bigham and Nordstrom 2000).



For most AMD waters, the pH varies between 0.5 and 4.0. In those conditions, aluminium is present as a free cation or/and as Al-sulphate complexes. Its concentration varies among acid pit lakes around the world, e.g. pit lakes from coal and lignite mining present different aluminium concentration from 0.01 to 4.0 mM (2.7-108 mg/L; Friese et al. 2013) whereas hard-rock metal mine pit lakes in the Iberian Pyrite Belt (IPB) present concentrations of 0.5-30 mM (13.5-810 mg/L; Sánchez-España et al. 2008a). Aluminium is also frequent in acidic mine drainage of the IPB, where concentrations up to 95 mM (2.6 g/L) can be found (Sánchez-España et al. 2005), in the San Telmo mine ~270 mM (7.3 g/L) of aluminium was measured in water of an acidic pool receiving pyrite leachate (March 2006; Sánchez-España et al. 2008b).

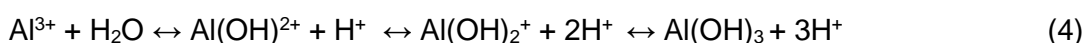
In several pit lakes of the IPB aluminium and its oxy-hydroxysulphates play an important role as a buffer system at pH values of 4.0-4.5, which is found near the lake bottom of meromictic lakes such as the Cueva de la Mora and Guadiana pit lakes (Sánchez-España et al. 2011, 2014b, Falagán et al. 2014). At pH <4.0, aluminium stays in solution, but over this pH value it starts to precipitate as felsöbányaite and/or as hydrobasaluminite as shown in equation 2 and 3, respectively. Hydrobasaluminite is

rapidly dehydrated to felsőbányaite; therefore, throughout this chapter it will be referred to felsőbányaite. At pH 5.2, the aluminium initially present in solution may precipitate by up to 96-98%, as observed in neutralization experiments conducted with acidic mining lake waters (Sánchez-España et al. 2011).

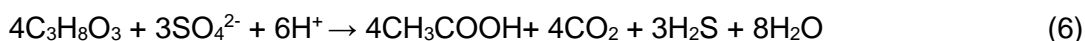
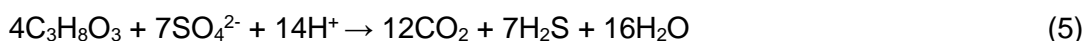


The formation of these colloidal compounds in the deep waters of acidic mining lakes causes a significant removal of aluminium, which is clearly visible in the vertical profiles of aluminium concentration (Table 6.2 and Table 2.2, Sánchez-España et al. 2011). In the absence of fine-grained particulate material and turbidity (which is characteristic of the clear, Fe(II)-rich deep waters of these lakes), bacterial cells could conceivably act as templates for the nucleation and crystalline growth of felsőbányaite and hydrobasaluminite precipitates. Such a bio-induced precipitation mechanism has been already described in neutral environments (Ferris et al. 1989, Konhauser and Urrutia 1999, Konhauser 2006), and Al precipitation around cells of sulphate-reducing bacteria has also been observed in German coal mine pit lakes (Meier et al. 2012).

When an aluminium hydroxysulphate or aluminium hydroxide is formed (pH >4.5) protons are released to solution as shown in the equations 2, 3 and 4.



Some species of acidophilic sulphate reducing bacteria (SRB) use glycerol ($\text{C}_3\text{H}_8\text{O}_3$) to reduce sulphate in proton consuming equations (Johnson and Hallberg 2009).



Therefore, these SRB can theoretically use the protons released during the formation of aluminium hydroxysulphates, thus favouring the precipitation of this metal.

This chapter explores the capacity of a bacterial consortium of acidophilic sulphate reducers to drive the precipitation of aluminium using a bench scale reactor. The bioreactor pH was set at 4.5-5.0 based on known chemistry of two pit lakes of the IPB. These lakes, Cueva de la Mora and Guadiana, have an upper oxic layer with pH values

between 2.6 to 2.9, while the anoxic bottom layers have pH values of 4.2-4.5. The middle layer, the chemocline, presents a gradient of pH from 2.5-2.8 to 4.0-4.2. Aluminium is a major component in the water column of these two pit lakes (Table 6.2), (Sánchez-España et al. 2008a, 2011, Falagán et al. 2014).

Table 6.2. Aluminium concentration in the water column of some acid pit lakes in the Iberian Pyrite Belt. ^a, Santofimia et al. 2012; ^b, Santofimia and López-Pamo 2013.

Pit lake	Depth (m)	pH	Al ³⁺ (mM)
Cueva de la Mora	6	2.6	4
	10.5	3.0	4.7
	30	4.5	2.5
Guadiana	7	2.9	2.6
	15	2.7	4
	55	4.2	5.6
San Telmo	5	2.8	4.4
	25	2.8	6
	90	2.9	4.8
Nuestra Señora del Carmen ^a	0	2.2	2.6
	15	2.2	9.2
Concepción ^b	1	2.5	0.3
	16	3.5	4.4

Therefore, the aim of this study was induce the precipitation of aluminium by increasing the pH of synthetic mine water via biosulphidogenesis, and also to identify the aluminium species formed during this precipitation process.

6.2 MATERIALS AND METHODS

6.2.1 BIOREACTOR SETTINGS

A 2.5 L bioreactor (Electrolab Ltd, UK) was used for these experiments. The reactor was half-filled with 1-2 mm diameter porous glass beads (Poraver Dennert GmbH, Germany). Sterile glass beads were inoculated with glass beads taken from a “mother

reactor” previously described by Ñancuqueo and Johnson (2012b). The bacteria consortium used comprised different bacterial species (Table 6.3) dominated by SRB and some other acidophilic bacteria able to grow under low pH anaerobic conditions.

The bioreactor (Fig. 6.1) working volume was established at 2.3 L coupled to a unit, which controlled pH, temperature, agitation (Electrolab 300, UK). Anoxic conditions were maintained by continuous bubbling with oxygen-free nitrogen. The pH of the reactor was set at 5.0; a pH electrode was connected to the 300 control unit that pumped feed liquor when the pH rose (as a consequence of sulphate reduction) above 5.0. Temperature was set at 30°C and was controlled by a temperature probe inside the reactor; an outside jacket embracing the vessel provided heat. The bioreactor content was stirred (50 rpm) to provide some mixing. To allow the removal and transfer of gases (CO₂ and H₂S) from the confined bioreactor, a jet of oxygen-free nitrogen was continuously bubbled through the reactor. Since H₂S (the toxic metabolic product of sulphate-reduction) is a volatile toxic compound, this was bubbled into a 50 mM copper sulphate solution (200 mL) in a receiving gas bottle (Fig. 6.1). Copper reacts with the H₂S producing black precipitates (CuS) which are readily harvested.

Table 6.3. Physiological characteristics of the acidophilic bacteria previously detected in the “mother reactor” (Ñancuqueo and Johnson 2012b and Santos et al. personal communication).

Isolate	Metabolism	Oxygen requirement	Reference
<i>Desulfosporosinus acididurans</i>	Sulphate-reducer	Anaerobe	Sánchez-Andrea et al. 2014
<i>Peptococcaceae bacterium CL4</i>	Sulphate-reducer	Anaerobe	Rowe et al. 2007
<i>Peptococcaceae bacterium CEB3</i>	Sulphate-reducer	Anaerobe	Ñancuqueo and Johnson 2012b
<i>Acidocella aromatica</i>	Heterotroph. Grows on organic acids and acetic acid under anaerobic conditions	Facultative anaerobe	Jones et al. 2013
<i>Acidithiobacillus ferrooxidans</i>	Iron/sulphur oxidizer. Iron reducer	Facultative anaerobe	Pronk et al. 1991, Ohmura et al. 2002
<i>Actinobacterium</i> sp. IR1	Heterotroph	Unknown	Ñancuqueo and Johnson 2012b
<i>Alicyclobacillus</i> sp. IR2	Undetermined	Unknown	Ñancuqueo and Johnson 2012b
<i>Clostridium</i> sp.	Heterotroph	Anaerobe	Chapter 4

The feed liquor was composed of 2-7 mM glycerol, 0.02 mg/L yeast extract, 0.7-2.2 mM aluminium sulphate and small amounts of other elements (Table 6.4). The pH of the feed liquor was necessarily lower (pH 2.5/3.0) than that of the reactor (pH 5.0).

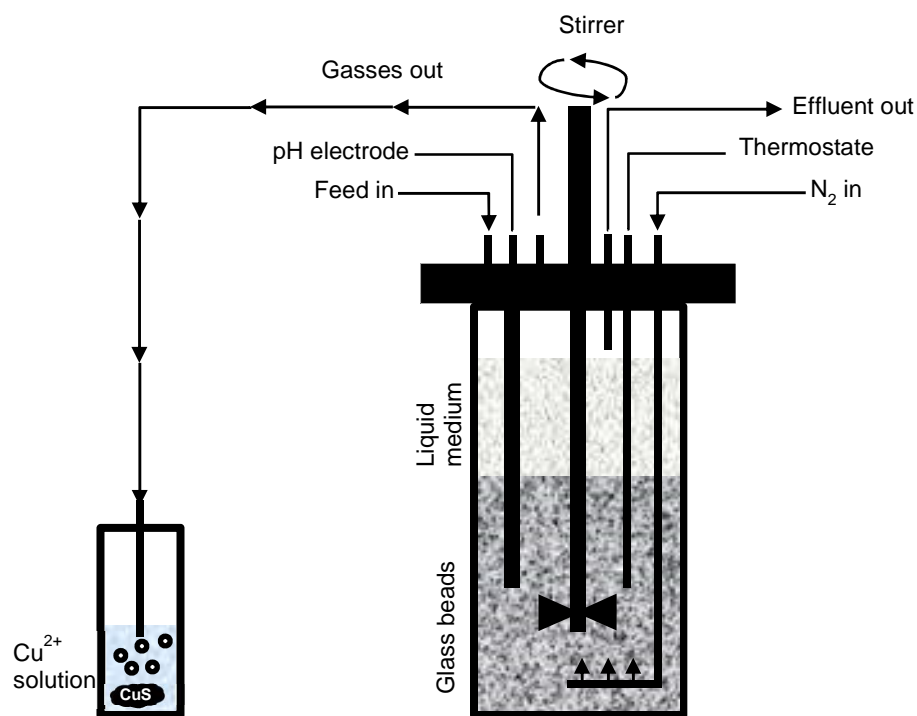


Figure 6.1. Schematic bioreactor setting (top) and photograph of the working reactor (bottom). Arrows indicate the liquid/gas flow.

6.2.2 ANALYTICAL METHODS

Aluminium was analysed by atomic absorption spectrometry (AAS) using a Varian SpectrAA 220 FS. Glycerol was analysed with a Dionex ICS 3000 ion chromatography system fitted with a Carbo Pac MA1 column and ED amperometric detector and acetate was measured using a Dionex IC25 ion chromatograph with an Ion Pac AS-11 column equipped with a conductivity detector at Bangor University (Ñancuqueo and Johnson 2012b).

Table 6.4. Composition of the feed liquor used in bioreactor experiments.

	Concentration	
	mM	mg/L
NH₄⁺	1.36	24.48
Na⁺	0.18	4.14
Cl⁻	0.13	4.61
Mg²⁺	0.41	9.96
PO₄³⁻	0.07	6.65
Ca²⁺	0.01	0.40
NO₃⁻	0.02	1.24
K⁺	0.2	7.82
Fe²⁺	0.1	5.58
SO₄²⁻	2.8-16.3	268.8-1564.8
Al³⁺	1.5-4.5	40.5-121.5
Glycerol	2-7	184-644
Yeast extract	-	0.2

Suspended particles were taken from inside the reactor and analysed by scanning electron microscopy (SEM) coupled with electron dispersion spectrometry (EDS). Compositional analyses were carried out on graphite-coated samples placed on carbon stubs using a JEOL JSM 7000F microscope at the SGIker facilities at the Basque Country University (UPV/EHU). Solid samples were also analysed by X-ray diffraction (XRD) using a PANalytical X'Pert Pro diffractometer operated with a continuous scan

range of 5-70° 2θ with Cu Kα radiation, 40 kV, 40 mA, graphite monochromator and an automatic slit.

6.2.3 BIOMOLECULAR ANALYSES

DNA was extracted from the membrane filters (Millipore 0.2 μm pore size) using MoBio “ultraclean soil DNA isolation kits”, following the manufacturer’s instruction. Bacterial 16S rRNA genes were amplified using the polymerase chain reaction (PCR) the primers used were the 27FC labelled with a fluorescent dye Cy5 in a cysteine (5’-3’ AGAGTTTGATCMTGGCTCAG; Lane 1991) and 1387R (5’-3’ GGGCGWGTGTACAAGGC; Marchesi et al. 1998).

Terminal restriction enzyme fragment length polymorphism (T-RFLP) analysis of amplified genes was carried out to assess the microbial diversity of samples. Amplified DNA was separately digested with the restriction enzyme HaeIII, the lengths of the gene fragments were determined using capillary electrophoresis (CEQ-Beckman Coulter), and the T-RFs (terminal restriction fragments) were compared with known T-RFs of the consortium species (Table 6.5).

Table 6.5. Terminal restriction fragments position with the restriction enzyme HaeIII of the isolates previously detected in the “mother reactor” (*Bangor Acidophile Research Team* database).

	T-RFs
<i>Desulfosporosinus acididurans</i>	214 ± 2
<i>Peptococcaceae bacterium CL4</i>	322 ± 2
<i>Peptococcaceae bacterium CEB3</i>	595 ± 2
<i>Acidithiobacillus ferrooxidans</i>	253 ± 2
<i>Acidocella aromatica</i>	228 ± 2
<i>Actinobacterium</i> sp. IR1	231 ± 2
<i>Alicyclobacillus</i> sp. IR2	215 ± 2
<i>Clostridium</i> sp.	221 ± 2

6.3 RESULTS

6.3.1 BIOREACTOR

The removal of the aluminium from the influent liquor was successful. The bacteria consortium used was usually grown in the absence of this metalloid in the “mother reactor”. Therefore, after a period of adaptation to the presence of aluminium (2 mM) bacterial activity started, evidenced by the production of acetic acid (Fig. 6.2) and H₂S (data not shown; equation 6), and also by the decrease of the hydraulic retention time (HRT; Fig. 6.3).

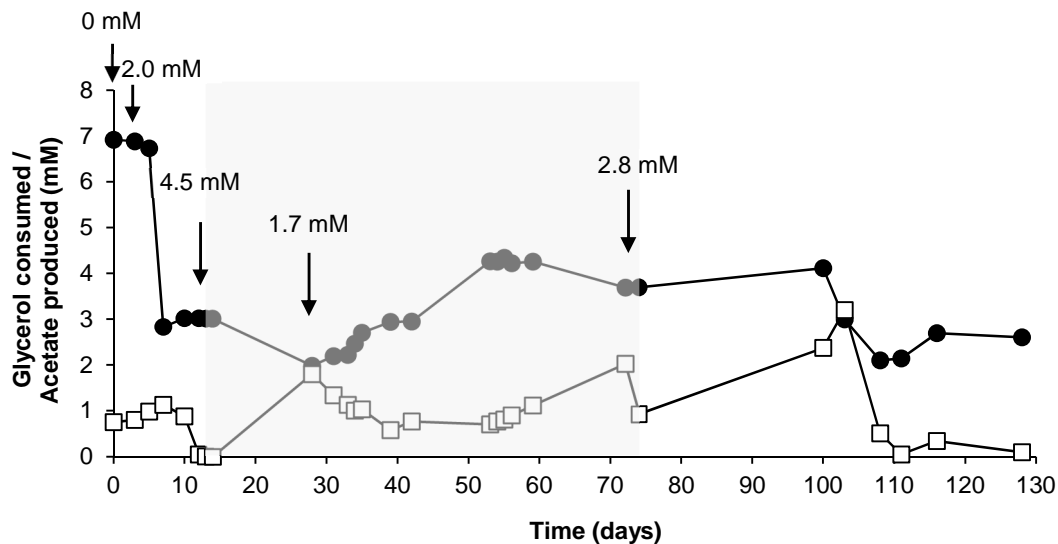


Figure 6.2. Changes in glycerol consumption (●) and acetate production (□). Downward pointing arrows indicate concentrations of aluminium in the feed liquor at different stages of the experiment. The feed liquor was pH 3.0, except during the shaded time phase (pH 2.5) and the bioreactor was maintained at pH 5.0 throughout.

Variations in the hydraulic retention times (HRT) were probably caused by fluctuations in bacterial activity. The HRT follows the slightly similar tendency than the aluminium precipitated (Fig. 6.3). Although the scatter plot (Fig. 6.4) shows the correlation $r=0.04$ between aluminium being precipitated (%) and HRT (h) in the whole experiment, the last 80 days of the experiment the correlation value was $r=0.5$ (data not shown). Thus, as Fig. 6.3 reflects, the higher the HRT, the more of aluminium was removed from solution, and vice versa.

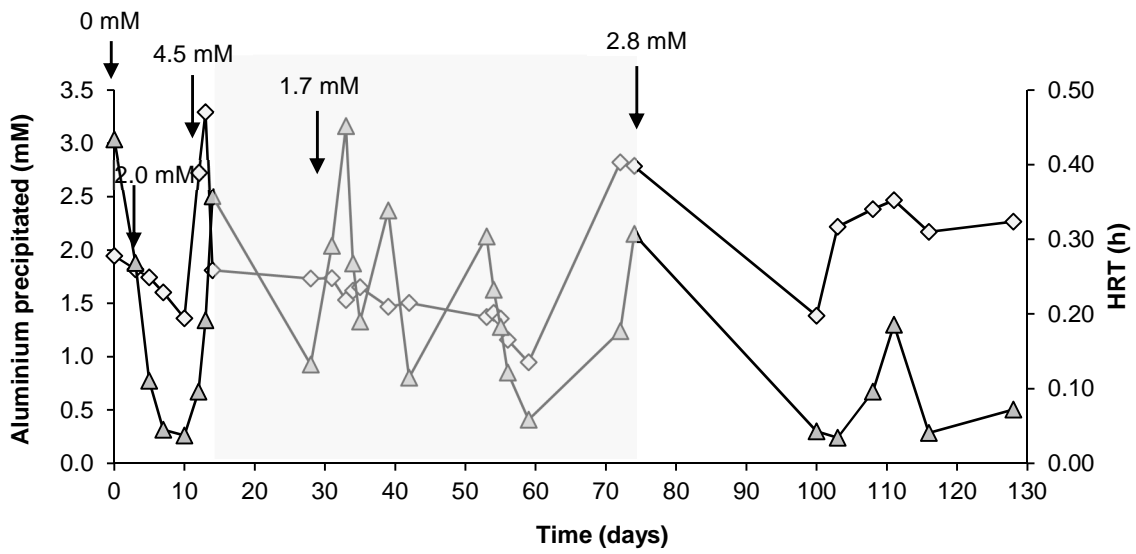


Figure 6.3. Hydraulic retention time (Δ ; HRT) and aluminium precipitation (\diamond) during the experiment indicating the different aluminium concentrations (mM; downward pointing arrows) used in the feed liquor at pH 3 and 2.5 (shadowed).

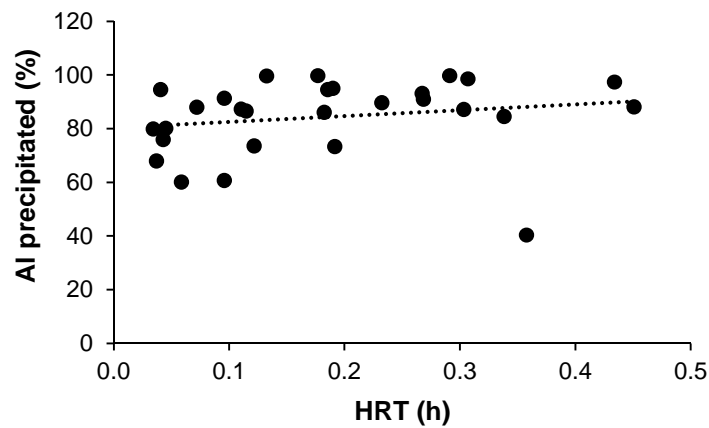


Figure 6.4. Scatter plot showing the correlation between hydraulic retention time (HRT) and aluminium precipitation (%) during the whole of the experiment (130 days).

In the last stage of the experiment, glycerol consumption was stabilised (Fig. 6.5), and, although HRT still varied (Fig. 6.3), aluminium was removed at a constant rate (>80%, Fig. 6.5).

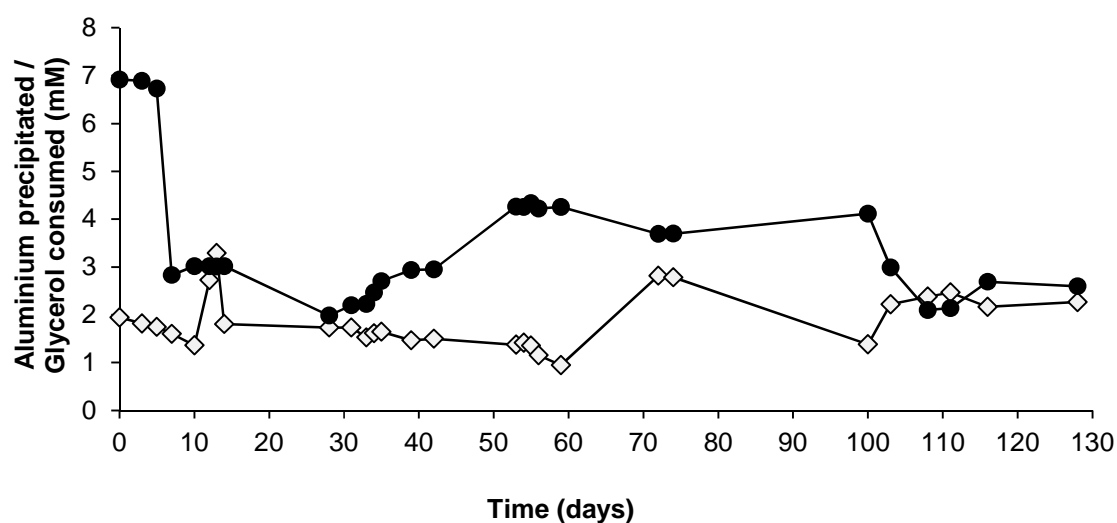


Figure 6.5. Changes in glycerol consumption (●) and aluminium precipitation (◇) throughout the experiment.

6.3.2 ANALYSIS OF PRECIPITATES

The SEM-EDS study of Al solids obtained in the bioreactor showed the presence of aluminium hydroxysulphates, e.g. felsőbányaite-like, hydroxybasaluminite-like, and alunite-like precipitates (Fig. 6.6, Fig. 6.7 and Table 6.6). However, it is often difficult to distinguish between felsőbányaite and hydroxybasaluminite as both are very similar materials, with different water contents (Bigam and Nordstrom 2000, Sánchez-España et al. 2006, 2011). XRD spectra of bioreactor-formed precipitates suggests hydrobasaluminite for nearly amorphous precipitates (Fig. 6.7). The formation of other sulphate salts was highly feasible, as the amount of sulphate in the feed liquor medium was very high (~8.3 to ~14.2 mM), and not all the sulphate was completely reduced or incorporated to the Al-hydroxysulphates. Other elements (e.g. P, Mg, Fe; Table 6.6) were present in the aluminium precipitates, which might be due to incorporation of these elements during the formation of the aluminium hydroxysulphates.

Bacterial cells were apparently covered by felsőbányaite and mixed with it (Fig. 6.6). Although, *a priori*, this could suggest nucleation of this mineral around the bacterial cells (bacterially mediated surface precipitation), no clear evidence of biomineralization was found within the reactor precipitates, in contrast to the findings of Meier et al. (2012).

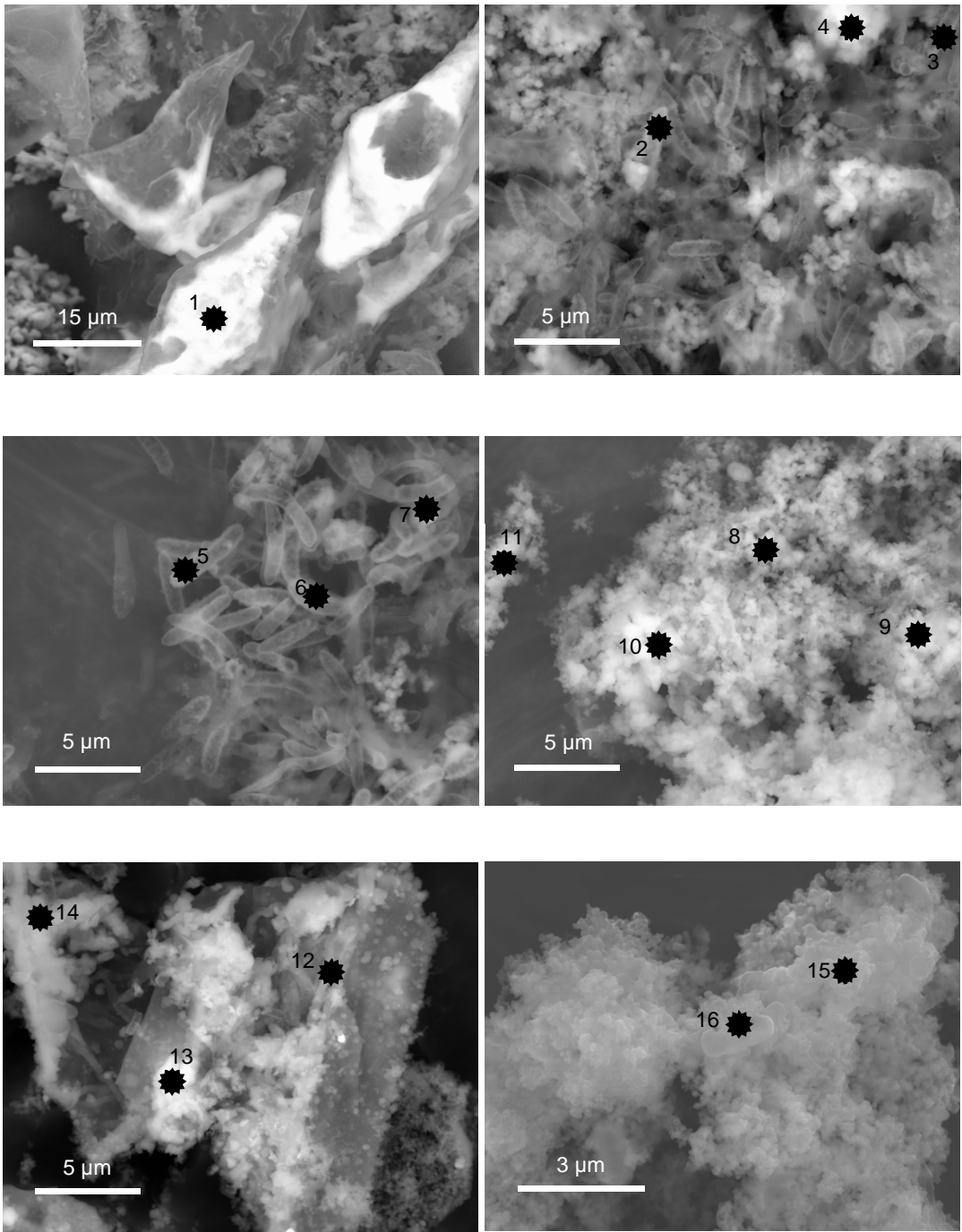


Figure 6.6. SEM images of bacterial cells and precipitates formed in the bioreactor collected along the experiment. Semi-quantitative chemical analyses (EDS) of points 1-16 are given in Table 6.6.

Table 6.6. Selected EDS analyses and possible mineralogy of the precipitates showed in Fig. 6.6. Gibs, gibbsite; Alu, alunite; Fsb, felsőbányaite; Hbs; hydrobasaluminite.

Site	O	Na	Mg	Al	Si	P	S	Cl	K	Ca	Fe	
	%	%	%	%	%	%	%	%	%	%	%	
Gibs*	34.59			34.59								
Alu*	54.08			19.54			15.48		9.44			
Fsb*	65.5			23.25			6.91					
Hbs*	72.01			16.75			4.98					
1	8.98			1.16	0.29		89.57					Native S
2	46.46			35.68		2.73	14.32		0.81			
3	37.39			39.69	0.58	3.87	17.51		0.97			
4	60.83			28.42	0.22	1.53	8.57		0.42			Fsb-like
5	70.28			22.87		1.16	5.68					Fsb-like
6	66.65			23.85		1.54	7.95					Fsb-like
7	67.38			22.76	0.46	2.04	7.36					Fsb-like
8	63.13	0.44	0.32	19.22	0.26	1.49	8.83		6.09		0.23	Alu/Fsb-like
9	63.03	0.4	0.31	18.4	0.25	1.45	9.17		6.98			Alu/Fsb-like
10	64.47	0.36		19.8	0.26	1.57	8.71		4.83			Alu/Fsb-like
11	70.72			18.07		1.47	6.77		2.98			Hbs-like
12	67.93			22.99	0.9	0.77	5.93		1.09		0.39	Fsb-like
13	67.27			24.31	0.71	0.71	6.44		0.56			Fsb-like
14	70.32	0.7	0.27	12.38	2.84		2.65	0.27	0.93	9.12	0.52	
15	68.61			22.76	0.62	0.69	5.06	1.56	0.25		0.25	Hbs/Fsb-like
16	67.57			23.04	0.59	0.99	5.29	1.87	0.27		0.27	Hbs/Fsb-like

* Theoretical percentages based on the ideal formulation of felsőbányaite, hydrobasaluminite, alunite and gibbsite (webmineral.com).

6.3.3 BACTERIAL BIOREACTOR POPULATION

During the first period of the experiment, the T-RFLP profile revealed the presence of several bacteria that had also been identified in the “mother reactor” (Table 6.5) such as *Clostridium* sp. or *Actinobacterium* sp. IR1, although, after a period of adaptation at the presence of aluminium, the activity of sulphate-reducing bacteria drove the reactor. T-RFLP analysis showed that the diversity of the bacterial community was mainly limited to three dominant microorganisms; *Desulfosporosinus (D.) acididurans*, *Acidocella (Ac.)*

aromatica and *Acidithiobacillus (At.) ferrooxidans* (Fig. 6.8). The sulphate-reducer *D. acididurans* was the most abundant during the time of the experiment, which is in good agreement with observation that glycerol was being effectively used to reduce sulphate (neither *Ac. aromatica* nor *At. ferrooxidans* can use glycerol as an electron donor). The other two microorganisms, which are known to be tolerant to high aluminium concentrations (Jones et al. 2013, Hedrich and Johnson 2013), are able to grow under anaerobic conditions. *Ac. aromatica*, a facultative anaerobe, uses acetate and produces hydrogen when grown under anaerobic conditions in co-culture with *D. acididurans* (Johnson and Hallberg 2009). *At. ferrooxidans* can oxidize both ferrous iron or sulphur and under anaerobic conditions and can use ferric iron as terminal electron acceptor in place of oxygen. However, no ferric iron was present in the influent liquor and it thought more likely that *At. ferrooxidans* was scavenging the small amounts of dissolved oxygen present in the feed liquor, which was not de-oxygenated.

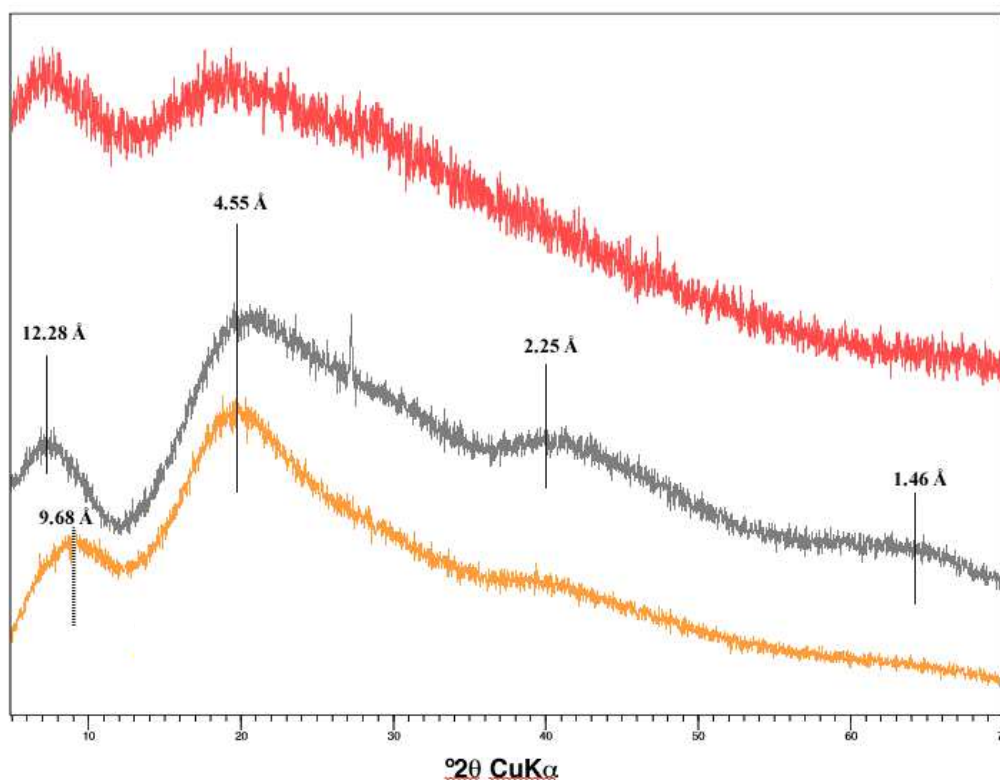


Figure 6.7. XRD pattern of hydrobasaluminite formed in the bioreactor (red) compared with similar precipitates obtained after a titration experiment with San Telmo pit lake water (Sánchez-España et al. 2011), felsőbányaite (yellow) and hydrobasaluminite (grey) formed at pH 4.8 and pH 5.2 respectively.

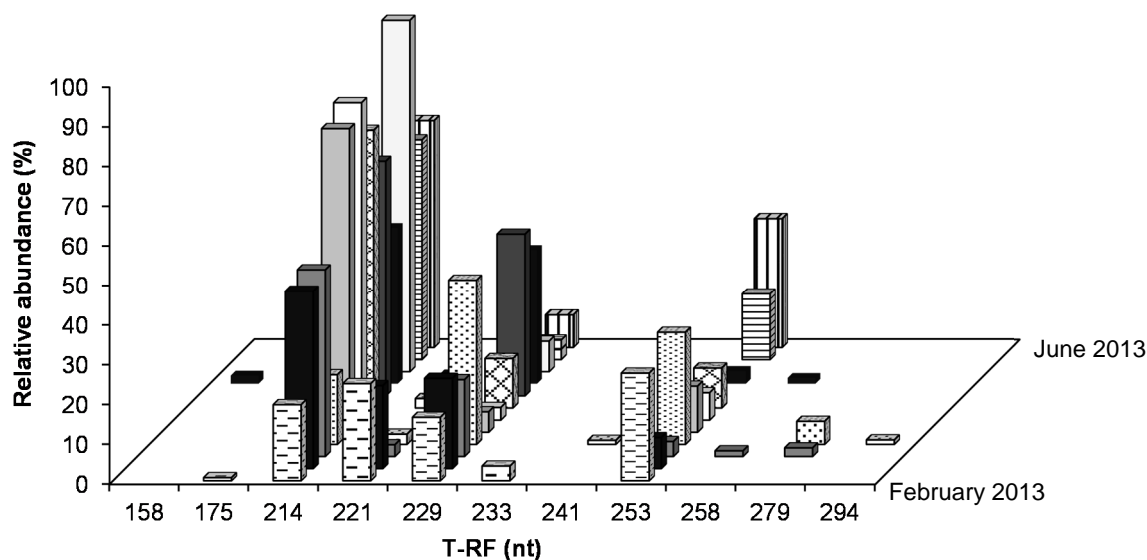


Figure 6.8. T-RFLP profiles of amplified bacterial 16S rRNA gene (digested with HaeIII) of filtrates from the liquor in the bioreactor. From the front to the back different samples analysed since February 5th to June 17th, 2013. T-RFs: 214, *Desulfosporosinus acididurans*; 229, *Acidocella aromatica*; 253, *Acidithiobacillus ferrooxidans*; 221, *Clostridium* sp.

6.4 DISCUSSION

Different aluminium concentrations were tested in the experiment. When the bacterial consortium was grown in the presence of aluminium, the sulphate-reducers used the protons released from the hydrolysis and precipitation of aluminium. For example, in the 35th day of the experiment, bacterial glycerol consumption was 2.7 mM (Table 6.7), 1.7 mM of which was completely oxidized to CO₂ and 1 mM was partially oxidized to acetate (equation 3). From equations 5 and 6, it was calculated that this amount of glycerol consumption requires 7.4 mM of protons. At that time, 1.65 mM of aluminium was precipitated. If felsőbányaite precipitation (equation 2) was considered as the main reaction driving the bioreactor, for 4 mM of Al precipitated, 10 mM of protons would have been generated; thus, every 1.65 mM of aluminium, 4.12 mM of protons would have been generated. When the feed liquor was more acidic (pH 2.5), the theoretical proton acidity present was 3.16 mM. Therefore, 7.3 mM of protons were available for the sulphate-reducers, which is nearly identical to the quantity of protons required for the glycerol consumption at that time. Thus, it can be concluded that the

sulphate-reducers used the protons that were released during aluminium precipitation and present in the low pH feeding liquor.

Table 6.7. Glycerol consumption, acetate production and aluminium precipitation at different feeding liquor conditions of pH, glycerol and aluminium concentration in the bioreactor.

pH	Glycerol	Al	Consumed	Produced	Precipitated	Generated ¹	Required ²
Inflow			Glycerol	Acetate	Al	Total H ⁺	Total H ⁺
	mM	mM	mM	mM	mM	mM	mM
3.0	3.02	2.0	3.02	0.88	1.36	4.4	8.81
3.0	3.02	4.49	3.01	0	3.29	9.23	10.54
2.5	3.00	1.74	2.23	1.14	1.53	6.99	5.51
2.5	3.00	1.74	2.7	1.04	1.65	7.29	7.39
2.5	4.4	1.58	4.25	0.77	1.41	6.69	13.34
2.5	3.7	2.83	3.7	2.03	2.82	10.21	8.86
2.5	3.7	2.83	3.7	0.93	2.78	10.12	11.08
3.0	2.15	2.61	2.1	0.51	2.38	6.95	6.33
3.0	2.15	2.61	2.14	0.05	2.47	7.17	7.39
3.0	2.61	2.58	2.6	0.1	2.27	6.66	8.9

¹, protons availability when considered those coming from the feeding liquor and those released when aluminium precipitated (equation 2).

², protons required taking account of equations 5 and 6.

Since aluminium was removed rapidly from solution, it was considered not to be of major toxicity to the bacterial cells. The increasing aluminium concentration in the outflow (after a period of high glycerol consumption, Fig. 6.5) was probably caused by the low HRT; the required time for the equation 2 (and 3) to occur was higher than the velocity with which the liquor was replaced.

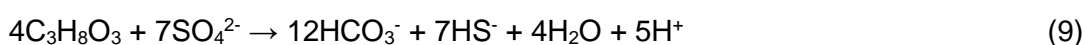
Selective removal of aluminium was possible by keeping a pH inside the bioreactor at about 5.0 and a hydraulic retention time with values between 0.12 and 0.23 h (Fig. 6.2 and Fig. 6.3). To precipitate 2 mM of aluminium would require a hydraulic retention time of ~0.25 h.

At different stages of the experiment, the amount of protons necessary to oxidize the consumed glycerol was higher than that generated by the aluminium precipitation and the low-pH feeding liquor (Table 6.7). The dominating microorganisms in the

bioreactor were *D. acididurans* and *Ac. aromatica*. As shown by Kimura et al. (2006) if glycerol is partially oxidized, acetate is produced (equation 6) and *Ac. aromatica* can use this acetate as carbon/energy source to grow under anaerobic conditions (equation 7) producing hydrogen. Then, *D. acididurans* uses that hydrogen as a second electron donor to reduce sulphate (equation 8).



In addition, at pH 5, HS^- and HCO_3^- are also present (equation 9).



This reaction usually occurs under higher pH conditions (pH >7), indicated by the pK_a of the equations 10 and 11 (pK_a : $\text{CO}_2/\text{HCO}_3^- \sim 6.3$; $\text{H}_2\text{S}/\text{HS}^- \sim 7$), than those prevailing in the bioreactor. This reaction could occur at acidic conditions if slight changes of pH take place in the reactor at a micro scale.



However at pH 5 the main SRB metabolic products are still CO_2 and H_2S rather than HCO_3^- and HS^- . As these processes are proton generating, more protons would be available to reduce sulphate, and that would give a final glycerol consumption and protons requirement higher than if only those generated by the aluminium precipitation and the low-pH feeding liquor were considered.

The aluminium tolerance of *Ac. aromatica* and *At. ferrooxidans*, has been previously studied showing a high tolerance, 200 and 300 mM respectively (5.4 and 8.1 g/L respectively), to the presence of aluminium (Jones et al. 2013, Hedrich and Johnson 2013b). The tolerance of other bacteria present in the “mother reactor” has not been studied yet, as in the case of *Actinobacterium* sp. IR1. A similar *Clostridium* sp. (strain MCF105) to that found in the “mother reactor” (16S rRNA genes sequences of both *Clostridium* presented 99% similarity) was found to be not very tolerant to the presence of aluminium (strain MCF105 grows under 1 mM (27 mg/L) but not at 5 mM aluminium (135 mg/L); Chapter 4). This is in accord with the disappearance of this bacterium when aluminium was added to the reactor feeding liquor. However, the dominant bacterium in

the bioreactor, *D. acididurans* is able to grow in the presence of 10 mM aluminium (270 mg/L; Sánchez-Andrea et al. 2014). The dominance of this bacterium in the bioreactor is probably due to the high pH (5.0) in the bioreactor and not caused by the presence of dissolved aluminium.

In acidic Al-rich waters, different bacterial metabolisms are slowed by the presence of this cation. For example, Ojumu et al. (2007) reported that aluminium reduces the growth rate of *Leptospirillum ferriphilum* at all concentrations tested. If aluminium were firstly removed from solution by precipitation, its negative effects would be reduced. The presence of aluminium ion causes a reduction of the quality of bio-hydrometallurgical operations; at high concentrations, this metallic ion may also cause inhibitory effects on iron oxidizers (Blight and Ralph 2008). Removing aluminium from the acidic solution is an advantage regarding the bacterial activity in bio-hydrometallurgical processes.

Moreover, the formation of Al-hydroxysulphates contributes to the removal of soluble trace metals (Lee et al. 2002). The low crystallinity of these Al-hydroxysulphates make them ideal sorbents of trace metals, e.g. Pb, Cu, U, Zn, which are readily adsorbed to these amorphous minerals (Sánchez-España et al. 2006). Therefore, favouring the formation of felsőbányaite in acid mine water would also facilitate the removal of other toxic elements.

A similar process to that demonstrated in this paper has been found to occur naturally in pit lakes of the IPB. A pertinent example is the Cueva de la Mora pit lake. In the water column of this pit lake, aluminium concentration decreases below the chemocline (Sánchez-España et al. 2009, 2011). This fact may be partially explained by the presence of sulphate-reducers in this layer (Falagán et al. 2014, Diez-Ercilla et al. 2014), which favours the consumption of protons by the aforementioned process, and thus help to increase the water pH, which in turn favours the precipitation of aluminium as Al-hydroxysulphates. Sánchez-España et al. (2011) showed that felsőbányaite and/or hydrobasaluminite are formed in acid mine waters. Moreover, similar white precipitates were found in a reactor under non-controlled conditions filled with lake sediments and water from the oxic layer of the Cueva de la Mora pit lake. After a month of incubation period, white precipitates (probably Al-hydroxysulphates) were observed on the water-sediment interface and aluminium in solution decreased an order of magnitude (Falagán et al. personal communication, Fig. 6.9). This was also interpreted as being mainly the result of the sulphate-reducing activity of the microorganisms inhabiting the sediments

of this lake (Falagán et al. 2014). The reactor sediment was analysed after 4 months of incubation and showed evident signs of evolution in the precipitates. The sediment of the first two centimetres was mainly composed by hydronium-jarosite (incorporating aluminium), goethite and detrital particles as quartz, muscovite and chlorite.

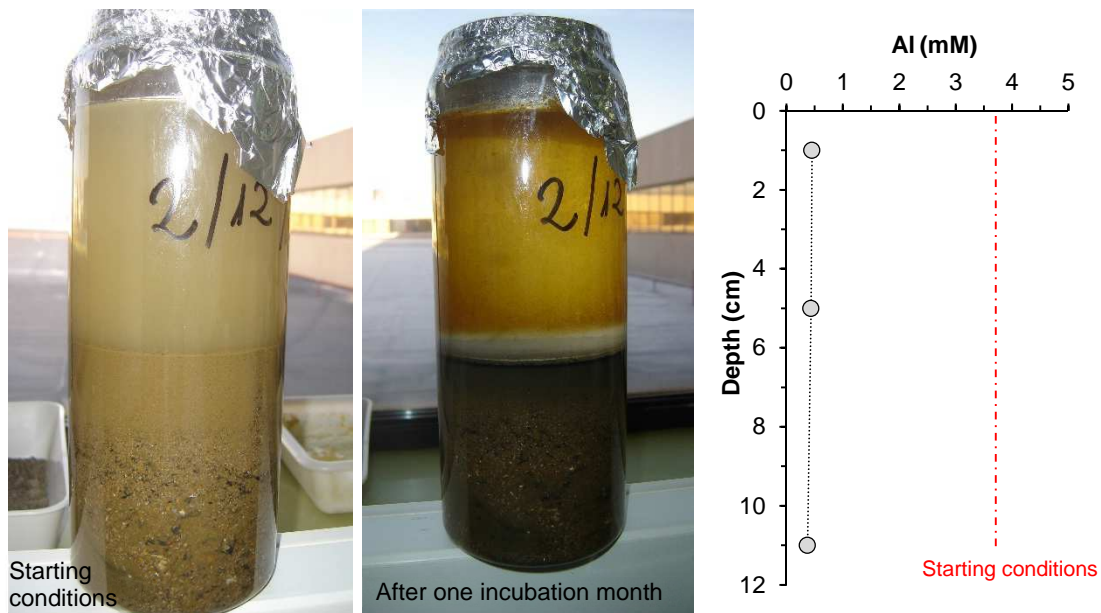


Figure 6.9. Non-controlled reactor of sediments and water from the Cueva de la Mora pit lake. The pictures illustrate the reactor sediments and water colour changes after one month of incubation, note the white layer formed in the second one. The graph shows the initial aluminium concentration (red dashed line) in the lake's shallow waters and the final concentration (black dotted line) measured in the reactor after 4 months of incubation.

6.5 CONCLUSIONS

Biologically-induced aluminium precipitation can be mediated by acidophilic and acid-tolerant strains of sulphate reducing bacteria that consume protons as part of their respiratory metabolisms, thereby causing solution pH to increase. Aluminium can be removed from acid mine drainage maintaining a slightly high pH biologically by sulphate reduction. No previous pH adjustment is necessary.

Although the bacterial composition of the bioreactor was determined pH, it was also conditioned by the presence of dissolved aluminium as some of the bacteria present in absence of aluminium disappeared after exposed to this metalloid.

Although bacteria seemed trapped within aluminium precipitates, no extra- or intra-cellular aluminium precipitation form has been identified as in Meier et al. (2012). This requires further investigation.

Aluminium precipitates formed in the sulphidogenic bioreactor as a result of pH increase induced by sulphidogenic bacteria, and that the precipitates in the reactor were identified and found to be similar to the found in the lake Cueva de la Mora, supporting the hypothesis that Al is precipitated in the lake waters at least in part due to the activities of sulphate-reducing bacteria.

**IMPLICATIONS OF
BIOLEACHING: ABIOTIC AND
MICROBIAL DISSOLUTION OF
CHALCOPYRITE**

7

A previous version of this chapter was submitted to *Minerals Engineering* journal (pending to be re-submitted).

7.1 INTRODUCTION.....	172
7.2 MATERIALS AND METHODS	173
7.2.1 MINERAL CONCENTRATE	173
7.2.2 MICROORGANISMS	174
7.2.3 LEACHING OF CHALCOPYRITE IN A pH- AND TEMPERATURE- CONTROLLED REACTOR VESSEL	174
7.2.4 FERRIC IRON LEACHING OF A COPPER (I) SULPHIDE (CHALCOCITE/DJURLEITE) MINERAL SAMPLE AT LOW REDOX POTENTIALS ..	175
7.2.5 ACID LEACHING OF A COPPER (I) SULPHIDE (CHALCOCITE/DJURLEITE) MINERAL SAMPLE IN THE ABSENCE OF FERROUS IRON	176
7.2.6 CHEMICAL ANALYTICAL METHODS.....	176
7.2.7 MINERALOGICAL ANALYSIS	177
7.3 RESULTS.....	177
7.3.1 ABIOTIC ANAEROBIC DISSOLUTION OF CHALCOPYRITE BY FERRIC IRON UNDER CONTROLLED REDOX POTENTIAL	177
7.3.2 MICROBIALLY-CATALYSED DISSOLUTION OF CHALCOPYRITE AT LOW REDOX POTENTIALS	179
7.3.3 BIOLEACHING OF CHALCOPYRITE UNDER LOW REDOX POTENTIALS IN THE PRESENCE OF ADDITIONAL FERROUS SULPHATE	180
7.3.4 MINERALOGICAL CHANGES IDENTIFIED IN THE BIOLEACHED CHALCOPYRITE CONCENTRATE	181
7.3.5 ABIOTIC LEACHING OF CHALCOCITE/DJURLEITE.....	181
7.4 DISCUSSION.....	183
7.5 CONCLUSIONS.....	186

Abstract

Chalcopyrite (CuFeS_2) is the most abundant copper-containing mineral in the lithosphere, but is known to be recalcitrant to bioleaching. Typically, only 20-30% of its copper content is leached by conventional oxidative bioleaching, though it has been shown that more efficient extraction can be achieved at elevated temperatures ($\sim 80^\circ\text{C}$), low redox potentials ($E_{\text{H}} \sim +600$ to $+650$ mV), in the presence of silver or chloride, and by galvanic interactions with other sulphide minerals such as pyrite. A chalcopyrite concentrate was leached under controlled aeration and controlled redox potentials ($+600$ to $+680$ mV) at 45°C and pH 1.5, both in the presence and in absence of defined microbial populations, and examined the speciation of iron under these conditions. The leaching of the chalcopyrite concentrate was found to be more effective in ferrous iron-rich media in the presence of iron- and sulphur-oxidizing bacteria where ferric iron was regenerated biologically. The significance of this finding to the understanding of the mechanism of the oxidative dissolution of chalcopyrite is discussed.

7.1 INTRODUCTION

Chalcopyrite is the most abundant copper-containing mineral in the lithosphere. The massive sulphide bodies in the Iberian Pyrite Belt (IPB) consist of dominant pyrite, associated with chalcopyrite, sphalerite, galena, and many other minor mineral phases (Velasco et al. 1998, Sáez et al. 1999). Chalcopyrite is found to be a common constituent of the ore in many mines of the area (Gaspar and Pinto 1991, Tornos 2006, Batista et al. 2007, Moura 2008, Grande et al. 2013). For example, chalcopyrite is the second most abundant ore mineral in the Cueva de la Mora ore deposit (Velasco et al. 1998, Sánchez-España 2000) and is also very abundant in the Las Cruces deposit, currently under exploitation (Yesares et al. 2015). It is being also widely present in mine tailings along the IPB (Álvarez-Valero et al. 2009); e.g. in the São Domingos mine, where some of the processing ore waste-water has up to 15% pyrite and chalcopyrite content (Álvarez-Valero et al. 2008).

The accumulation of tailings and waste dumps in mining areas is the cause of pollution of many water bodies, rivers, streams and soils all over the world. In the southwest of Spain (Huelva) it is possible to follow several contaminated acid mine drainages and outflow streams of a mining pit lakes that end in rivers that discharges into the sea. A better processing of minerals would generate less amount of waste materials and less contaminating sources.

The leaching of chalcopyrite is a major issue within mining industries and the scientific community. To date, a method allowing to completely extract all the copper still needs to be developed. Chalcopyrite is recalcitrant with respect to bioleaching (Watling 2006, Li et al. 2013). Typically, less than 30% of copper is solubilized from chalcopyrite using conventional oxidative biomining processing, and recovery rates in bio-heaps and run-of-mine dumps can be even smaller.

There is general consensus that this is due to the formation of passivation layers that accumulate on the surface of the mineral during bioleaching, though the nature of the passivating material(s) has been a topic of considerable debate and disagreement. Secondary ferric iron (jarosite-like) precipitates, elemental sulphur, polysulphides and metal-deficient sulphides (which essentially describe the same materials), and the secondary copper minerals chalcocite and covellite, have all be implicated as the agents of chalcopyrite passivation, though many of these have been demonstrated, at least

under certain conditions, not to hinder bioleaching of this mineral (e.g. d'Hugues et al. 2002, Holmes and Crundwell 2013). However, bioleaching of chalcopyrite has been shown to be more effective at elevated temperatures (~80°C; d'Hugues et al. 2002, Cordoba et al. 2008b), lower redox potentials (~+630 to +650 mV vs. SHE; Gericke et al. 2010, Cordoba et al. 2008a), in the presence of silver, either added extraneously (e.g. Cancho et al. 2007, Cordoba et al. 2008b) or when present in a mineral concentrate (Johnson et al. 2008), in chloride-supplemented liquors (Kinnunen and Puhakka 2004, Lu and Dreisinger 2013), when in contact with pyrite as in the Galvanox™ process (Nazari et al. 2011) and following regrinding of the passivated mineral (Li et al. 2013). However, no single theory has been suggested which accounts for why these disparate approaches appear to circumvent, as least to some degree, the problem of chalcopyrite passivation.

Chalcopyrite is a covalent copper mineral (Li et al. 2013) in which the oxidation state of copper is +1, iron +3 and sulphur -2 (Pierce et al. 2006). Schippers and Sand (1999) classified it as an “acid soluble” sulphide mineral which implies that it is subject to proton (hydronium ion)-catalysed dissolution in anoxic acidic liquors as well as being, in common with other metal sulphides, susceptible to oxidation by ferric iron. Schippers and Sand (1999) also described two mechanisms to account for the (bio)leaching of sulphide minerals (the polysulphide and thiosulphate mechanisms) which, as their names imply, result in the production of different sulphur species.

We have carried out a series of experiments on abiotic and bacterially-accelerated dissolution of a chalcopyrite concentrate at 45°C and pH 1.5, in which we have monitored the solution chemistry (leachate), including iron speciation. Our data show that, when (bio)leaching is carried out at relatively low redox potentials in the presence of ferrous iron, better results of leached copper are obtained. The aim of this study was therefore to improve our knowledge about the biotic and abiotic mechanisms involved in chalcopyrite leaching.

7.2 MATERIALS AND METHODS

7.2.1 MINERAL CONCENTRATE

A chalcopyrite concentrate containing ~30% copper was obtained from a mine in South America. It contained 83% chalcopyrite, 4% bornite, 3% each of pyrite, chlorite

and amphibolite, and 1% plagioclase. Chalcocite and covellite were each present at concentrations <1%. The elemental composition of the concentrate is shown in Table 7.1. The 80% of the concentrate had a particle size of 0.044 mm.

Table 7.1. Elemental composition (as wt.% concentrate) of the chalcopyrite concentrate used in the experiments is described.

Sulphur	34
Copper	29.7
Iron	29
Silicon	1.87
Calcium	10.7
Magnesium	0.6
Aluminium	0.26
Nickel	0.2
Cobalt	0.07
(Oxygen)	(3*)
All others	<0.02

*estimated value.

7.2.2 MICROORGANISMS

Two species of moderately thermophilic, acidophilic bacteria were used in bioleaching tests. These were the autotrophic sulphur-oxidizer *Acidithiobacillus (At.) caldus* (DSM 8485^T) (Hallberg and Lindström 1994) and the facultatively autotrophic iron/sulphur-oxidizer *Sulfobacillus (Sb.) thermosulfidooxidans* (DSM 9293^T). As well as oxidizing iron, *Sb. thermosulfidooxidans* is a facultative anaerobe that can respire on ferric iron (Johnson and Hallberg 2009). Both bacterial species have been previously detected in the IPB (the Tinto river) using prokaryotic microarrays probes (Garrido et al. 2008).

7.2.3 LEACHING OF CHALCOPYRITE IN A pH- AND TEMPERATURE-CONTROLLED REACTOR VESSEL

A bioreactor (Electrolab, UK) with a 2 L working volume reactor vessel was used in all experiments. The temperature was maintained at 45°C, and pH at 1.5 (by automated

addition of sulphuric acid or sodium hydroxide), and the vessel was stirred at 150 rpm. Redox potentials were maintained, where necessary, via a combination redox electrode coupled to a purpose-made (Electrolab) redox-control unit (a meter coupled to a pump that added either sterile ferric sulphate or air to the reactor vessel, depending on the experimental set up). All solutions were heat-sterilized, but no other attempts were used to maintain sterility. Samples were withdrawn from the reactor at regular intervals to measure pH and E_H values off-line, and the on-line electrodes were recalibrated when necessary (some drift of redox potentials measured within the reactor occurred during the long-term experiments). Anaerobic conditions were imposed where necessary by bubbling oxygen-free nitrogen through the reactor liquors.

Several experiments were carried out, some in which the reactor was inoculated with a mixed culture of the acidophilic bacteria, and others where bacteria were not added. In the latter, redox potentials were maintained by automated addition of ferric sulphate to the reactor liquors, while in the inoculated experiments the set redox potential limits were maintained by controlled microbial regeneration of ferric iron (which was reduced to ferrous when oxidizing chalcopyrite) by regulating aeration of the bioreactor. In all tests, sterile basal salts solution (2 L in abiotic experiments and 1.9 L in the bioleaching tests), containing 3.4 mM $(\text{NH}_4)_2\text{SO}_4$, 0.46 mM $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$, 0.67 mM KCl, 2.03 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.37 mM KH_2PO_4 and 0.06 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, was pre-heated to 45°C and adjusted to pH 1.5 in the reactor vessel, and then 50 g of (non-sterilized) chalcopyrite concentrate was added. One hundred millilitres of a mixed *At. caldus*/*Sb. thermosulfidooxidans* was added immediately following the chalcopyrite in bioleaching tests. In addition, ferrous sulphate was added (at 50 or 130 mM, final concentration) to the reactor in two bioleaching experiments. In both set ups, the slurry was stirred for a few minutes after the addition of the concentrate and a sample withdrawn to measure concentrations and speciation of copper and iron off-line. The reactors were sampled intensively during the first day of each experiment, and at longer intervals as the experiment progressed, for up to 50 days.

7.2.4 FERRIC IRON LEACHING OF A COPPER (I) SULPHIDE (CHALCOCITE/DJURLEITE) MINERAL SAMPLE AT LOW REDOX POTENTIALS

An experiment was carried out to examine copper speciation during abiotic leaching of museum-grade “chalcocite” (obtained from a commercial supplier) by ferric iron at low redox potentials. X-ray diffraction (XRD) indicated that most of the copper in the sample

was present as djurleite ($\text{Cu}_{1.94}\text{S}$) rather than chalcocite (Cu_2S), though in both of these minerals copper is present as Cu(I) . Ten grams of finely-ground sample of museum-grade mineral was placed in a 250 mL conical flask, and 100 mL of an acidic (pH 1.5) sterile ferrous/ferric sulphate solution (containing 104 mM ferrous iron and 4 mM ferric iron) and basal salts (pH 1.5) added. The measured E_{H} of the solution (prior to addition of the minerals) was +630 mV. Using the Nernst equation, this corresponds to an E^0 value of the ferrous/ferric couple of +713 mV, which is in the range of that typically found in very acidic sulphate-rich solutions (Welham et al. 2000, Johnson et al. 2012). Samples were removed at regular intervals to determine concentrations of copper (I) and total soluble copper. Additional ferric sulphate was added sporadically to increase the redox potential of the leach liquor, though this remained more negative than the starting value throughout the experiment.

7.2.5 ACID LEACHING OF A COPPER (I) SULPHIDE (CHALCOCITE/DJURLEITE) MINERAL SAMPLE IN THE ABSENCE OF FERROUS IRON

Two experiments, under oxic and anoxic conditions, were carried out to examine copper speciation during acid abiotic leaching of djurleite ($\text{Cu}_{1.94}\text{S}$). Five grams of finely-ground sample of the mineral was placed in a 250 mL conical flask, and 100 mL of an acidic (pH 1.5) sterile solution, and completely free of ferrous or ferric iron sulphate. Anoxic conditions were made by bubbling oxygen-free nitrogen into the sterile solution. Samples were removed at regular intervals to determine concentrations of copper (I) and total soluble copper.

7.2.6 CHEMICAL ANALYTICAL METHODS

Concentrations of copper (I) were determined using the colorimetric method described by Anwar et al. (2000). Concentrations of total copper were determined using the same technique, following reduction of copper (II) by hydroxylamine, and copper (II) concentrations determined from the differences. Ferrous iron was determined using the ferrozine assay (Stookey 1970), and total soluble iron on the same solution following reduction with hydroxylamine. The amount of elemental sulphur in mineral residues was determined using the colorimetric assay described by Hazeu et al. (1998), which involved extracting sulphur from samples of harvested (by centrifugation) residue with acetone, and measuring the amount of sulphur in solution by cyanolysis.

7.2.7 MINERALOGICAL ANALYSIS

Samples of bioleached chalcopyrite were removed from the bioreactor and examined using scanning and transmission electron microscopy coupled to energy dispersive X-ray spectroscopy (SEM-EDS, TEM). EDS semi-quantitative compositional analyses were carried out using a JSM-7000F electron microscope. Two drops of sample dispersed in ethanol were placed on double-sided adhesive carbon tape on SEM stubs and coated with graphite. TEM examination of an ethanol-dispersed solid residue samples was carried out using a Philips CM200 microscope. X-ray diffraction (XRD) was used to compare the mineralogy of non-processed and bioleached mineral concentrate. This was carried out using a PANalytical X'Pert Pro diffractometer operated with a continuous scan range of 5-70° 2 θ with Cu K α radiation, 40 kV, 40 mA, graphite monochromator and an automatic slit. All these mineralogical analyses were carried out at the SGIker facilities of the University of the Basque Country (UPV/EHU, Bilbao). In addition, mineralogical (XRD) and chemical (X-ray fluorescence, XRF) analyses of the copper (I) mineral sample were carried out at the laboratory of the Geological Survey of Spain (IGME).

7.3 RESULTS

7.3.1 ABIOTIC ANAEROBIC DISSOLUTION OF CHALCOPYRITE BY FERRIC IRON UNDER CONTROLLED REDOX POTENTIAL

The results from experiments carried out using low redox potential leaching of the chalcopyrite concentrate in the non-inoculated reactor are shown in Fig. 7.1 and Fig.7.2. These plots depict the three distinct phases of mineral dissolution that were observed: (i) an initial rapid increase in soluble metals via acid dissolution; (ii) a relatively rapid phase of chalcopyrite dissolution following the initial addition of ferric iron; and (iii) a protracted relatively slow later phase.

Fig. 7.1 (top) shows changes in total iron and copper concentrations, and speciation of iron, during the first five hours following addition of the concentrate to the reactor vessel, and before any addition of ferric sulphate. Soluble metals accumulated rapidly during the first several minutes, probably as a result of the dissolution of very fine particles produced during milling of the ore. The soluble iron was present as ferrous

throughout this phase, the redox potential of the concentrate leached remained at $<+580$ mV during this time.

Figure 7.1 (bottom) shows that copper increased in solution very soon after the controlled addition of ferric iron was initiated. This caused the solution E_H to be increased to $\sim+620$ mV (and it was subsequently maintained at $<+700$ mV for the duration of the experiment) and resulted in the dissolution of chalcopyrite, which had essentially come to a halt, to restart. Most of the ferric iron added to the reactor during this phase, and during the remainder of the experiment, was reduced to ferrous iron.

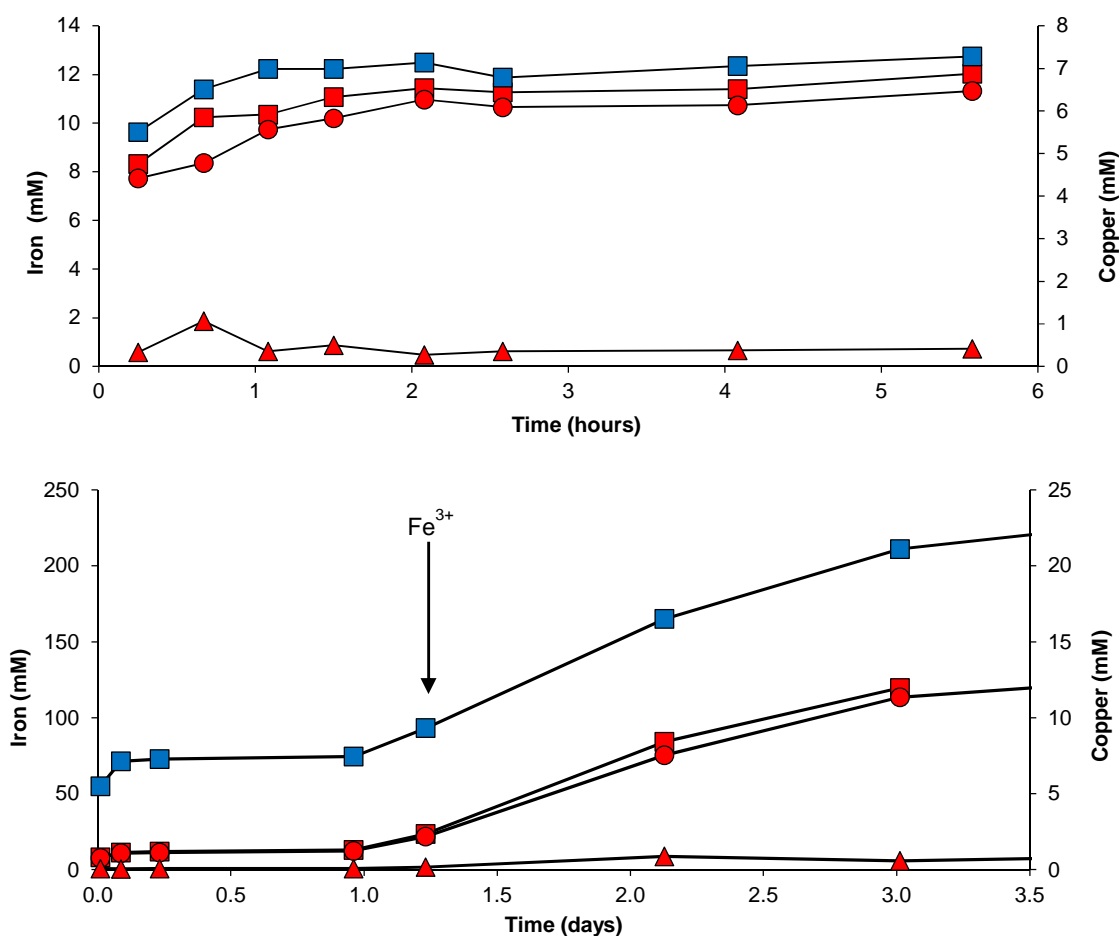


Figure 7.1. Abiotic leaching of a chalcopyrite concentrate under controlled redox potentials during the first phase (acid dissolution only) (top); changes in copper speciation induced by the initial addition of ferric sulphate (arrowed) (bottom). ■, total soluble copper; ●, sulphur; ■, total soluble iron; ●, iron (II); ▲, iron (III).

Changes in metal concentrations and speciation throughout the 20 day experiment are shown in Fig. 7.2. From day 8 to day 20 the E_H of the reactor liquor was allowed to increase from +630 mV to +680 mV. This caused the ratio of ferric to ferrous iron to increase. It is also shown that elemental sulphur accumulated, to ~50 mg/g mineral residue, in the abiotically-leached concentrate.

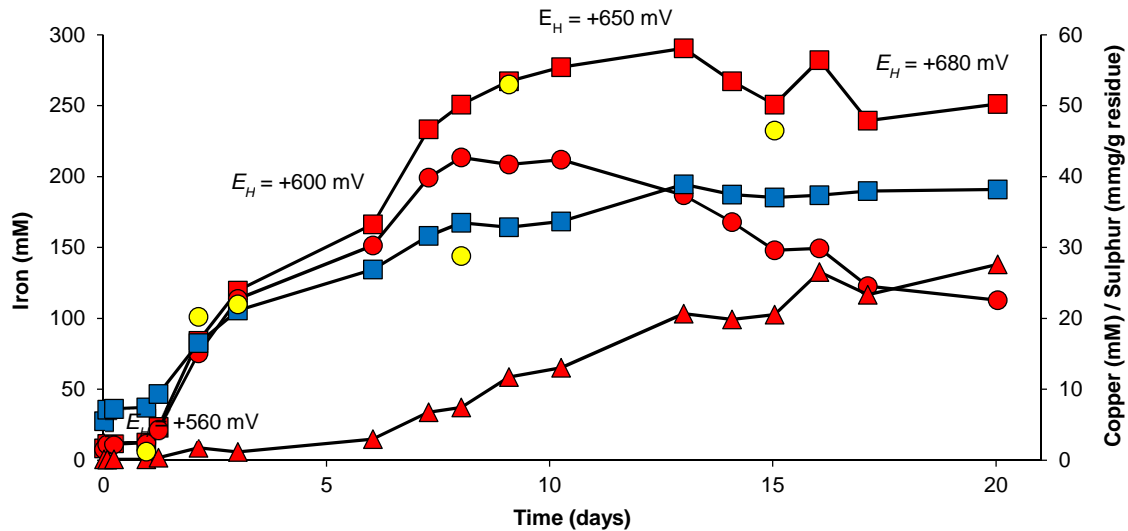


Figure 7.2. Abiotic leaching of a chalcopyrite concentrate under controlled redox potentials during the full-term experiment data. ■, total soluble copper; ●, sulphur; ■, total soluble iron; ●, iron (II); ▲, iron (III).

7.3.2 MICROBIALY-CATALYSED DISSOLUTION OF CHALCOPYRITE AT LOW REDOX POTENTIALS

Biological regeneration of ferric iron avoided having to add substantial quantities of ferric sulphate to maintain the target redox potential of the leach liquor, and also provided a mechanism for removing the sulphur that accumulated during oxidation of the concentrate. Results from a representative bioleaching experiment carried out at 45°C, pH 1.5, and an E_H of <+640 mV are shown in Fig. 7.3.

The regeneration of ferric iron was mediated in these experiments by *Sb. thermosulfidoxidans*, and E_H was maintained by controlling the aeration of the bioreactor vessel. It was noted that there was some initial accumulation of elemental sulphur in the mineral residue but that this declined to relatively low levels between days 2 and 4, presumably due to bacterial oxidation (mediated by *At. caldus*). Elemental

sulphur began to accumulate again in the mineral residue after day 7, reaching ~16 mg/g residue by day 16, and its presence at this time was confirmed by XRD analysis of a sample taken from the reactor. Re-inoculation of the reactor on day 16 with an active culture of *At. caldus* caused the elemental sulphur concentration to decline once more, reaching <1 mg/g mineral residue by day 32. Interestingly, the removal of this potentially passivating sulphur did not improve the rate of chalcopyrite dissolution.

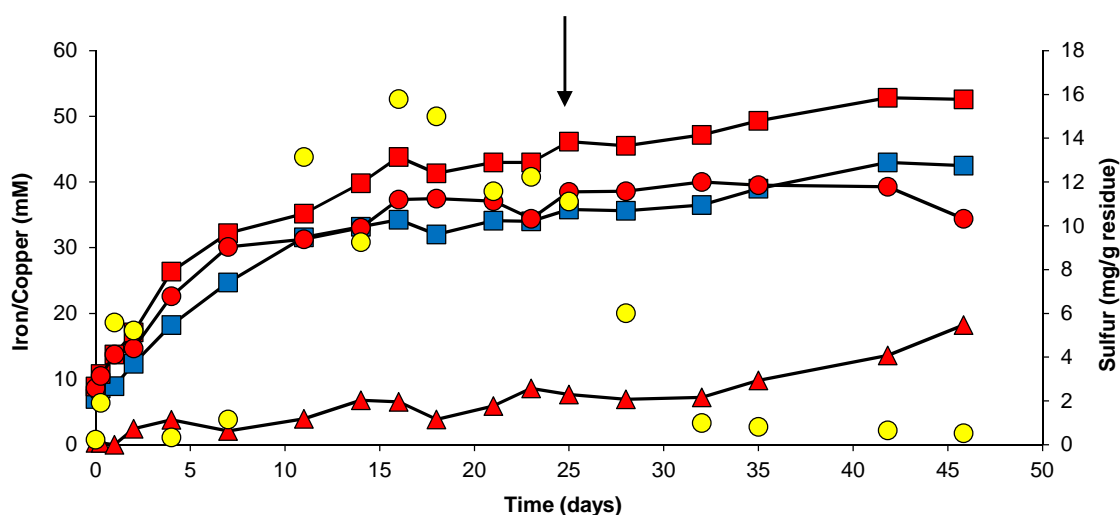


Figure 7.3. Changes in concentrations of copper and iron, and sulphur in the mineral residue, during the bioleaching of a chalcopyrite concentrate at low redox potentials. ■, total soluble copper; ●, sulphur; ■, total soluble iron; ●, iron (II); ▲, iron (III). The arrow indicates the point at which the bioreactor was re-inoculated with an active culture of *At. caldus*.

7.3.3 BIOLEACHING OF CHALCOPYRITE UNDER LOW REDOX POTENTIALS IN THE PRESENCE OF ADDITIONAL FERROUS SULPHATE

Addition of ferrous sulphate to the bioreactor liquor at the start of an experiment resulted in an improvement in copper leaching (Fig. 7.4, which shows data from an experiment in which 50 mM ferrous sulphate was added), in contrast to bioleaching experiments where no additional iron was added (Fig. 7.3).

Elemental sulphur in the mineral residue increased rapidly for the first 3-5 days and stayed at 20-25 mg/g residue in this experiment (Fig. 7.4), in contrast with the previous

experiment where elemental sulphur declined to values close to 1 mg/g residue (Fig. 7.3).

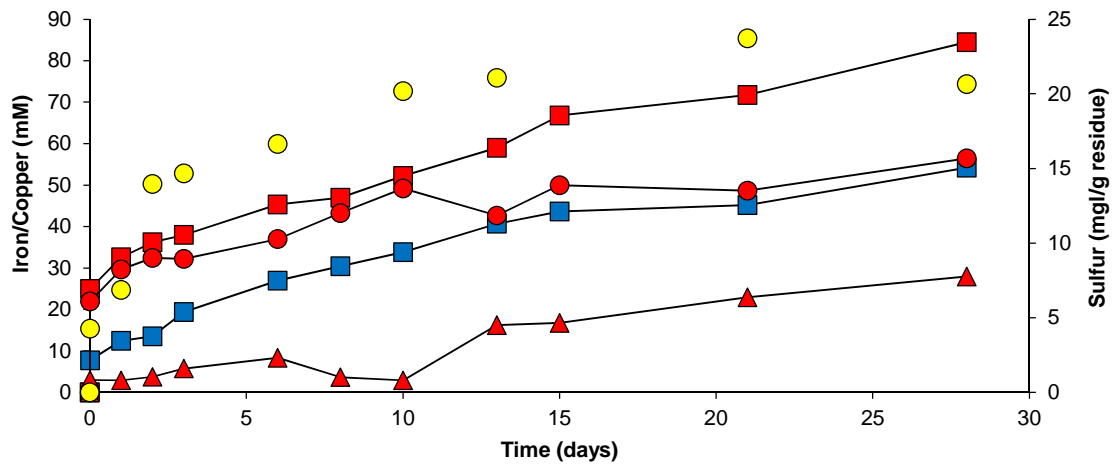


Figure 7.4. Changes in concentrations and speciation of copper and iron during the bioleaching of a chalcopyrite concentrate amended with 50 mM ferrous sulphate at day 0. ■, total soluble copper; ●, sulphur; ■, total soluble iron; ●, iron (II), ▲, iron (III).

7.3.4 MINERALOGICAL CHANGES IDENTIFIED IN THE BIOLEACHED CHALCOPYRITE CONCENTRATE

The bioleached chalcopyrite concentrate showed obvious evidence of oxidative dissolution in an acidic liquor, such as irregularly corroded surfaces with sharp curved edges, dissolution pits and other typical textural features. Some secondary minerals were identified by XRD and/or TEM, including irregular and banded aggregates of Fe-sulphates or oxy-hydroxysulphates, sub-idiomorphic grains of K-jarosite, thin films of amorphous silica and elemental sulphur (Fig. 7.5). However, there was no evidence of secondary copper sulphides in the bioleached residue.

7.3.5 ABIOTIC LEACHING OF CHALCOCITE/DJURLEITE

Copper leaching began immediately after the mineral was placed in the ferrous/ferric iron solution, and was further enhanced by intermittent addition of ferric sulphate (Fig. 7.6). As predicted by the range of redox potentials measured (+520 to +630 mV), ferrous iron was the dominant form of soluble iron throughout this experiment. In the iron-free acid leaching of djurleite/chalcocite the copper leaching also began immediately after the mineral was placed into the solution as if in the presence of iron. Similar concentrations

of copper were leached in both experiments (oxic and anoxic) and copper (II) was the dominant form. However, in a second experiment in completely anoxic and iron-free acid leaching conditions copper (I) was found to be up to 21% of the total copper, while redox potential was +454 mV (data not shown).

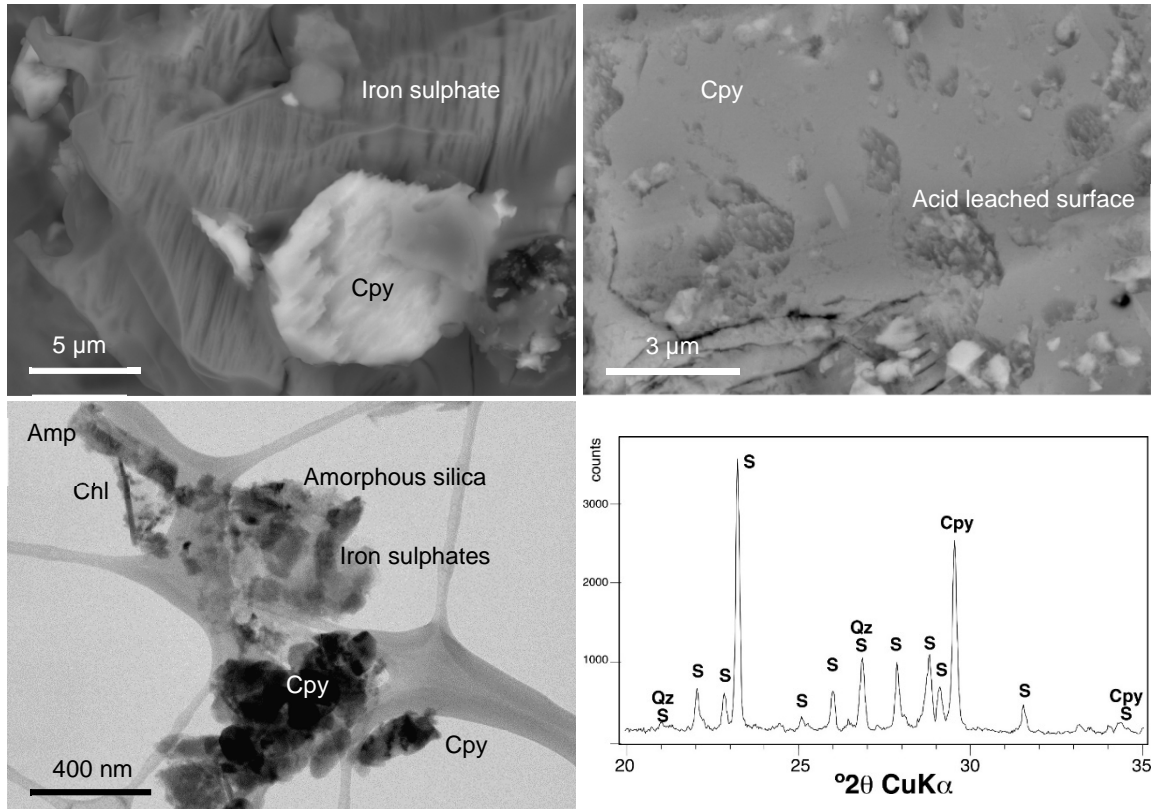


Figure 7.5. Mineralogy of the leached chalcopyrite showing dissolution features and the formation of iron sulphates (top and left bottom) and amorphous silica (left bottom) and native sulphur (XRD pattern showing characteristics reflections of chalcopyrite and native sulphur) in the leached chalcopyrite concentrate (right bottom). Mineral abbreviations: Cpy, chalcopyrite; Chl, chlorite; Amp, amphibole; S, native sulphur; Qz, quartz.

Using the colorimetric assay described by Anwar et al. (2000) copper (I) was identified as the main form of soluble copper during the abiotic leaching of the djurleite/chalcocite mineral sample in the presence of ferrous/ferric iron. These results differ with those obtained in the absence of ferrous/ferric iron. Therefore, it is reasonable to assume that iron interferes with copper in the assay and only copper (II) is present at the redox potential of the experiments (+520 to + 630 mV).

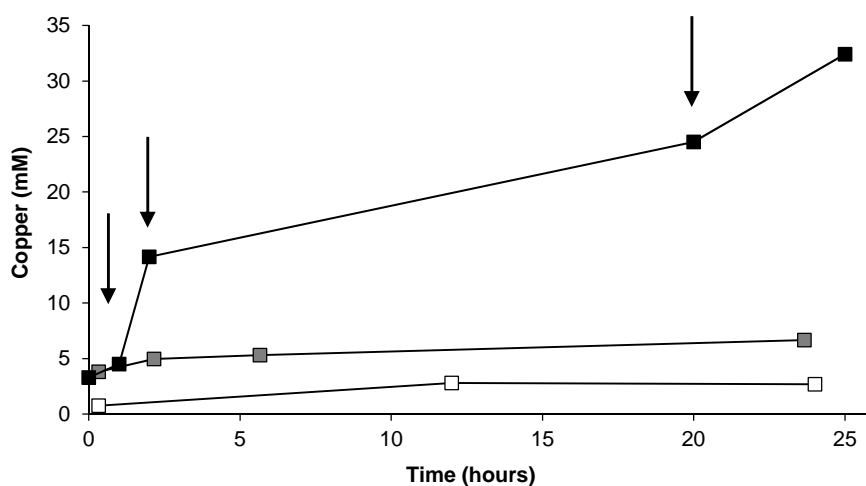


Figure 7.6. Total copper in solution of acid leaching of djurleite/chalcosite mineral under oxic and anoxic conditions. ■, in the presence of ferric iron, the pointing arrows indicate times at which additional ferric sulphate was added to the leach liquor; ▣, under oxic conditions in the absence of iron; □, under anoxic conditions in the absence of iron.

7.4 DISCUSSION

Previous reports of chalcopyrite bioleaching invariably indicate that soluble copper is present exclusively in leach liquors in its most oxidized form, copper (II) (e.g. Watling 2006). This assumption is based on large differences in published E^0 values of the iron (III)/iron (II) couple (+770 mV) and that of copper (II)/copper (I) (+340 mV). These figures are based on the premise that oxidized and reduced species of both metals are present in solution as non-complexed ions. In the case of iron in bioleachates generated by the oxidative dissolution of sulphide minerals, ferric iron is complexed both by sulphate (as $\text{Fe}(\text{SO}_4)^+$ and $\text{Fe}(\text{SO}_4)_2^-$) and by hydroxyl ions (as $\text{Fe}(\text{OH})_2^+$ and $\text{Fe}(\text{OH})_2^{2+}$; Welham et al. 2000) which results in the E^0 values of the ferrous/ferric couple being significantly less positive ($\sim +690$ to $+730$ mV; Welham et al. 2000; Johnson et al. 2012) than when ferric iron is not complexed. Conversely, the E^0 value of the copper (II)/copper (I) couple has been found to be significantly more positive than +340 mV in acidic sulphate-rich liquors (Johnson personal communication). These observations imply that, in low redox solutions ($\sim +600$ mV to $+650$ mV) all four ions (Cu^{2+} , Fe^{3+} , Cu^+ and Fe^{2+}) can coexist. This hypothesis is supported by Matocha et al. (2005) who reported that copper (II) could be reduced to copper (I) by ferrous iron at pH values of 5.2 to 5.5. On the basis of these reports, the rationale of the current work was to test the hypothesis that (i) soluble copper

present in leachates generated by low redox potential bioleaching of chalcopyrite comprises both copper (I) and copper (II), and (ii) the effect of ferric or ferrous iron on the leaching of a chalcopyrite concentrate.

Acid dissolution of chalcopyrite in an anoxic ferric iron-free liquor would cause the initial release of the three component elements in the same oxidation states as they occur in the mineral (equation 1):



In solution, these products would be subject to four competing reactions:

(i) oxidation of copper (I) by ferric iron (equation 2):



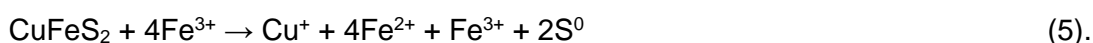
(ii) oxidation of hydrogen sulphide by ferric iron (equation 3):



(iii) precipitation of copper (I) sulphide (equation 4; ferrous sulphide is acid-soluble and does not precipitate at the low pH used in the current experiments):



and oxidation of chalcopyrite by ferric iron (equation 5):



Addition of the concentrate to the reactor vessel resulted in very rapid (within 10 minutes) dissolution of some of the minerals present in both abiotic and bioleaching experiments. Most of the soluble iron was present at this stage as ferrous. Concentrations of soluble iron were much greater than those of copper in this early phase, probably due to the dissolution of the acid-soluble ferrous iron mineral chlorite ((Mg,Fe,Al)₆(Si,Al)₄O₁₀(OH)₈), which would also have contributed to ferrous iron being the dominant soluble form of iron during the first few hours of the abiotic experiments. Acid-catalysed mineral dissolution continued for 2-3 hours, during which time the non-inoculated reactor was operated in anoxic mode, but then declined significantly. Between 1 and 3 hours after the start of the experiment, ferrous continued to be the dominant soluble form of iron present throughout the entire time frame of all (abiotic and

bioleaching) experiments. Redox potentials were not controlled in the abiotic experiments during this period but remained below +580 mV. Elemental sulphur accumulated during the acid dissolution phase (to ~2 mg/g mineral residue), indicating that chalcopyrite was also being oxidized by the ferric iron released from the acid dissolution of the mineral (equation 5).

Only very limited acid dissolution of the chalcopyrite concentrate was observed, but controlled addition of ferric iron (in the abiotic experiments) re-started the process. Again soluble iron was predominantly present as ferrous, as expected when considering that the redox potential was not allowed to increase above +620 mV from day 1 to day 8 (abiotic experiments). During this period, ferrous iron would have been produced mostly by the oxidation of chalcopyrite by the ferric iron added to the reactor (equation 5), though other reactions (equations 2 and 3) could also have contributed to the ferrous iron pool.

The fact that four moles of ferric ion are required to oxidize one mole of chalcopyrite and that only one mole of ferric iron is released from the mineral in equation (5) explains the requirement for continued provision of ferric iron, either added from an external source (as in the abiotic experiments) or regenerated biologically (as in the bioleaching experiments; Third et al. 2000) for the process to continue. However, it was also noted that chalcopyrite dissolution was both more rapid and more extensive (up to 60% of the copper present in the concentrate was leached) in ferrous sulphate-amended reactors.

Elemental sulphur accumulated in the mineral residue during abiotic (acid- and ferric iron-catalysed) dissolution, and also during the first few days of bioleaching. Most of this sulphur was removed initially by the bacteria present in the reactor, but a second phase of sulphur accumulation began after day 7, and the bioreactor needed to be re-inoculated (with *At. caldus*) to slowly eliminate the sulphur. A similar observation was reported by Xia et al. (2010). It is interesting to note that the removal of sulphur did not result in enhanced bioleaching of the concentrate, suggesting that it had a minor, if any, role in passivating chalcopyrite. This hypothesis was supported by bioleaching experiments carried out in reactors amended with ferrous sulphate, where more elemental sulphur accumulated in the mineral residue, but chalcopyrite oxidation was more effective.

The method used to determine the copper speciation was ineffective in the presence of ferrous iron as revealed on the acid leaching of djurleite/chalcocite mineral in the absence of ferrous/ferric iron as no soluble copper (I) was detected. When copper (I) binds to the reagent in the presence of high Fe(II) concentration, the equation (2) is

favoured towards the formation of copper (I). Therefore, it has been impossible to determine the copper speciation during the chalcopyrite (bio)leaching. Whether copper (I) is present during leaching or is extremely rapidly oxidized to copper (II) is still under discussion. Recent experiments showed some interesting results where copper (I) has been detected during acid leaching under strictly anoxic conditions in the absence of iron. This findings suggests that copper (I) is released during the acid leaching and rapidly oxidized to copper (II) in the presence of oxygen. However, if copper (I) is certainly present, it may be also present during the chalcopyrite leaching, and thus influence on the dynamics of this process.

Although copper speciation was impossible to determine, there are some interesting facts suggesting that copper species have an important role on the leaching of chalcopyrite. For example, Hiroyoshi et al. (2001) reported that chalcopyrite dissolution could be enhanced by adding ferrous iron and copper (II) to leach solutions at similar concentrations. This can be explained by the reaction between these ions (at low redox potentials), which produces copper (I) and ferric iron, which catalyses the oxidative dissolution of the mineral. Both chalcocite (Cu_2S) and covellite (CuS) have been detected as minerals formed during the bioleaching of chalcopyrite (as reported in Li et al. 2013). In theory, both of these can form as secondary minerals from the products of chalcopyrite dissolution (chalcocite from Cu^+ and H_2S , and the covellite from Cu^{2+} and H_2S). Bioleaching at low redox potentials, where Cu^+ is more prevalent, would favour the formation of the more readily oxidized mineral chalcocite.

7.5 CONCLUSIONS

As reported previously in literature the presence of ferric iron favours chalcopyrite leaching. When redox potential is kept at low values ($\sim +650\text{mV}$), ferric iron can be regenerated in the presence of an iron oxidizer (e.g. *At. caldus*). In which case, oxygen availability would serve as a control of the redox potential. Contrary to what has been reported in literature, the removal of elemental sulphur from the chalcopyrite grains does not enhance the copper leaching.

Theoretically, the presence of copper (I) in solution would favour the leaching of chalcopyrite. Whether or not copper (I) is present in chalcopyrite leachates remains unknown. The method reported by Anwar et al. (2000) to measure copper revealed to be ineffective to determine copper speciation in the presence of ferrous iron since copper

(I) is overestimated as ferrous iron reduces effectively copper (II) to copper (I) during the performance of the assay.

More research is currently being carried out in order to discern the role of copper and iron species in the leaching of chalcopyrite to achieve high copper recovery.

**GENERAL
CONCLUSIONS**

8

Cueva de la Mora and Guadiana (Herrerías mine) pit lakes are two complex acidic mine pit lakes located in the Iberian Pyrite Belt. The water column of both lakes is divided in two main layers separated by a transition layer with sharp redox potential and pH gradients. These three layers present different physico-chemical conditions, which evidences the existence of three different environments. In addition, sediments found at shallow depths and in the lake bottom also present different physico-chemical features which determine the inhabiting microbial community.

The microbial community of these pit lakes is distributed differently among the three layers. Photosynthetic algae and heterotrophic bacteria populate the upper layer. The microalgae are found at a depth where the photosynthetically active radiation (PAR) is <2% of that measured close to the lake surface. Apparently, the upper oxic layer of the lakes is poor in inorganic phosphorus as it is bound to ferric iron and transported downwards to the deep layers. In the transition layer, both, ferric and ferrous iron are present and free-inorganic phosphorus is more easily available to be used by microorganisms. This free-phosphorus diffuses through the transitional layer to the upper layer being available for the photosynthetic algae (Fig. 8.1). Therefore, algae growth is basically determined by phosphorus availability. In conclusion, phytoplankton is growing at a depth where there is enough of this nutrient to support cell growth being adapted to grow under such low PAR.

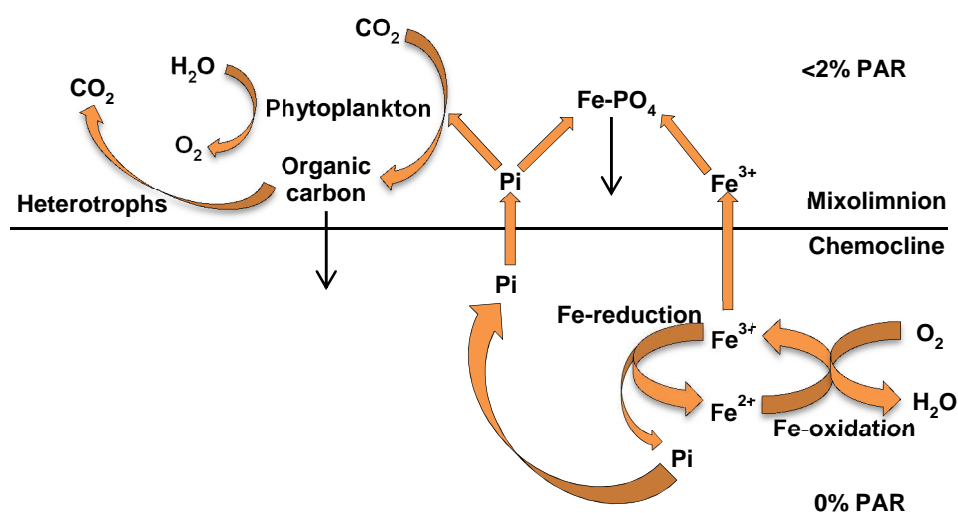


Figure 8.1. Schematic representation of the inorganic phosphorus (Pi) cycle and carbon showing the interconnection between the transition layer (chemocline) and the oxic layer (mixolimnion). PAR, photosynthetically active radiation.

Sulphur and iron biogeochemical cycles (Fig. 8.2) drive the redox chemistry of the transition layers in Cueva de la Mora and Guadiana pit lakes. Sulphur and iron oxidizers as well as sulphate and iron reducers are the dominant metabolisms in these zones. The availability of oxidized and reduced forms of substrate in these transition layers and the import of organic carbon from the upper layers makes this layer a very favourable environment for many bacterial species.

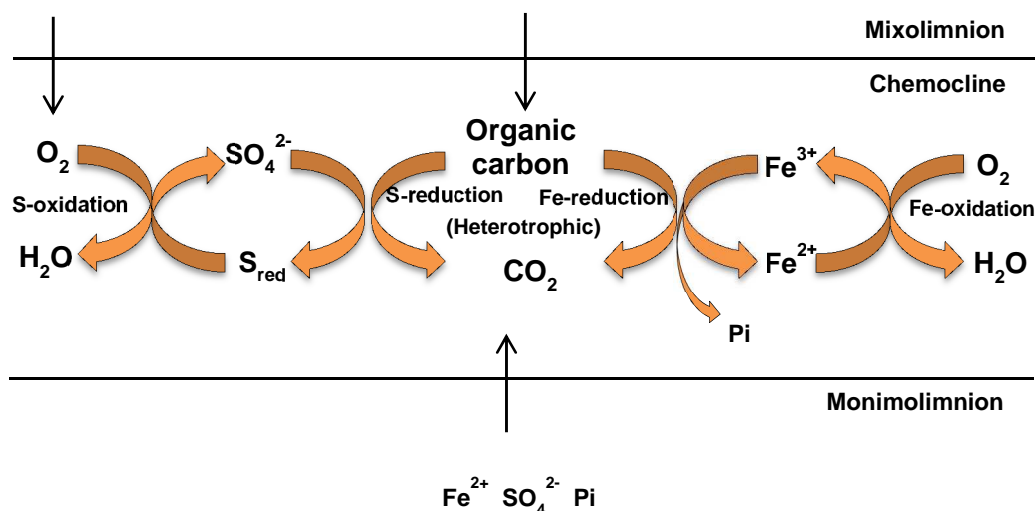


Figure 8.2. Schematic representation of the sulphur and iron cycles in the transition layer (chemocline) and the interconnection with the oxic layer (mixolimnion) and the anoxic layer (monimolimnion). Pi, inorganic phosphorus.

In contrast, no bacteria has been detected from the monimolimnion of both lakes (although one isolate was obtained from the monimolimnion of the Cueva de la Mora pit lake, this will be considered as anecdotal). This could be caused by either low organic carbon availability or by metal toxicity imposed by the extreme chemical composition of the lake waters. However, archaea was detected in the monimolimnetic waters of Cueva de la Mora.

Sediments were revealed to be populated by both archaea and bacteria. In bottom lake sediments in Cueva de la Mora the bacterial community is dominated by heterotrophic anaerobic bacteria whereas, the bacterial community in the shelf shore sediments is more diverse as iron and sulphur oxidizers and iron and sulphate reducers have been also found. In addition, the presence of some archaeal groups typically found in non-acidic environments (chemolitho-autotrophic ammonium-oxidizing and

methanotrophic archaea) have been detected in sediments of this lake revealing that the microbial community in the lake sediments is complex and more research needs to be done.

More than a hundred *Bacteria* were isolated from both lake waters (Guadiana and Cueva de la Mora) and sediments (Cueva de la Mora). Although many of them could be affiliated to known acidophilic bacterial species, twelve new species and two new genera were discovered: four acidobacteria, six firmicutes and three proteobacteria. Among all of them, six isolates (five aerobes and one anaerobe) were characterized. Five new species are proposed and one new genera: "*Acidicapsa ferrireducens*", "*Acidicapsa acidophila*", "*Granulicella acidophila*", *Acidibacter ferrireducens* (already published in Falagán and Johnson 2014) and "*Clostridium acididurans*", and a new genera *Acidibacter* sp. (Falagán and Johnson 2014) have been proposed. All these strains showed different metal tolerances. All the aerobic isolates are tolerant to elevated aluminium concentration (75-300 mM) whereas the anaerobe is able to grow under the presence of >10 mM arsenic (III) and arsenic (V). In addition, all of them are very sensitive to copper, which concentration is very low in the lake waters. Moreover, *Acidibacter ferrireducens* appeared to have extra-cellular iron precipitates when growing under the presence of high ferrous iron concentration. The possible accumulation of iron precipitates by this new acidophilic bacterium should be studied in the future.

Although nitrate and ammonium are both present in the lake waters (nitrate is slightly more abundant in the mixolimnion and ammonium is notably more abundant in the monimolimnion), neither denitrification nor nitrification have been detected in any of the water samples. The limit for nitrification has been established by Jeschke et al. (2013) at pH ~3.0. However, the lake waters present higher pH (~4.5) at depth and pore waters in sediments also show pH >3.0, more investigation should be carried out in order to discern if any kind of ammonium-oxidizers and nitrate reducers are present in these acidic environments.

Bio-induced aluminium precipitation can be easily achieved using a bacterial consortium dominated by sulphate-reducing bacteria in a bioreactor. Aluminium can be removed from acid mine drainage maintaining a slightly high pH biologically by sulphate reduction. This phenomenon is also occurring in the chemocline waters in Cueva de la Mora. If enough organic carbon is provided to the lake water, sulphate-reduction could be induced, and precipitation of toxic dissolved metals would be achieved in order to

generate less contaminated waters. This should be studied as a possible bioremediation strategy to improve the water quality of these lakes and consider its possible implementation in the future.

More than 50% of the copper present in a chalcopyrite concentrate has been leached during the experiments. Chalcopyrite leaching was optimized by controlling redox potential by regenerating ferric iron in the presence of an iron oxidizer (e.g. *Acidithiobacillus caldus*). In which case, oxygen availability would serve as a control of the redox potential. Contrary to which has been reported in literature, the removal of elemental sulphur from the chalcopyrite grains does not enhance the copper leaching. There is still much to be investigated to find the best way to extract all the copper. The profits obtained from the extracted copper from mine tailings would be enough to cover the economic cost of the process and could also help to remediate at least, part of the contamination produced in the Iberian Pyrite Belt by mining activities.

As a concluding remark, this study shows that geomicrobial interaction play a pivotal role in the water quality (redox chemistry, metal content, acidity) of the acidic pit lakes formed in abandoned metal mines. Detailed investigations on the microbial community inhabiting these environments and its interaction with their surrounding aqueous environment (metal ions, organic compounds, mineral particles) help to understand the geochemical dynamics of these water bodies. The knowledge emerging from these geomicrobiological investigations can be very useful not only for bioremediation purpose, but also for biotechnological applications, which can be important in the future for the modern mining industry.

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9

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