DIVERSIDAD MICROBIANA DE LOS SEDIMENTOS ANAEROBIOS DE RÍO TINTO

Irene Sánchez-Andrea

UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE BIOLÓGICAS

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DIVERSIDAD MICROBIANA DE LOS SEDIMENTOS ANAEROBIOS DE RÍO TINTO

Irene Sánchez-Andrea

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Dr. José Luis Sanz y Dr. Ricardo Amils, catedráticos del Departamento de Biología Molecular de la Universidad Autónoma de Madrid,

CERTIFICAN:

Que los trabajos de investigación desarrollados en la memoria de tesis doctoral: "DIVERSIDAD MICROBIANA DE LOS SEDIMENTOS ANAEROBIOS DE RÍO TINTO" son aptos para ser presentados por Irene Sánchez-Andrea ante el Tribunal que en su día consigne, para aspirar al Grado de Doctor en Microbiología por la Universidad Autónoma de Madrid.

V°B° Director Tesis

Dr. José Luis Sanz

V°B° Director Tesis

Dr. Ricardo Amils

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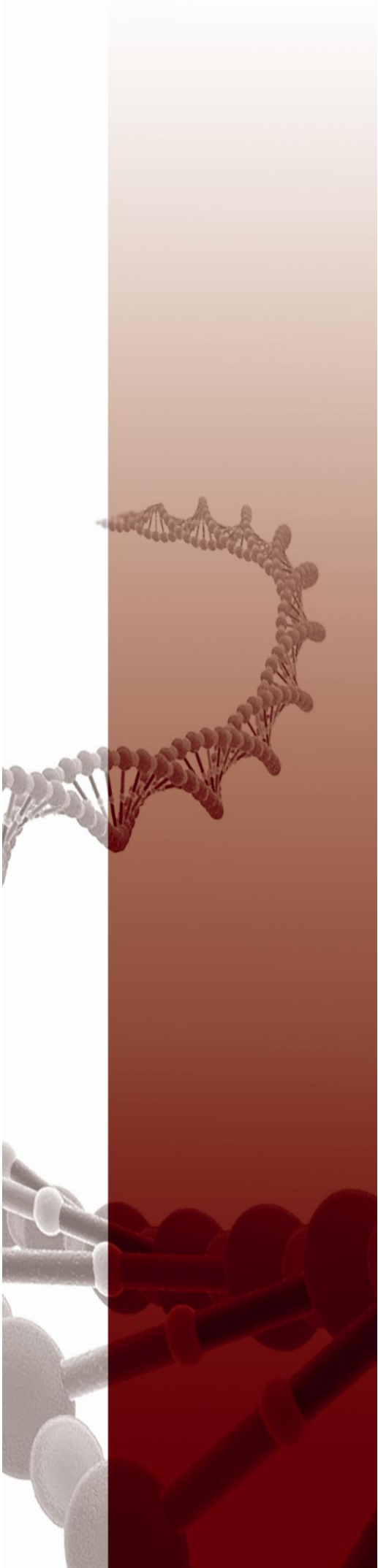

El río Tinto (Huelva, España) es un ambiente natural, semejante a los denominados drenajes ácidos de mina, producido por la bio-oxidación de sulfuros metálicos procedentes de la Faja Pirítica Ibérica. Hasta ahora, y a pesar de su interés ecológico, sus sedimentos habían sido escasamente estudiados. En esta tesis, presentamos un amplio estudio de la microbiota anaerobia de los sedimentos del río Tinto combinando técnicas dependientes e independientes de cultivo siguiendo el llamado ciclo completo del rRNA.

Usando técnicas de biología molecular, ha sido desarrollado un modelo geomicrobiológico de los distintos ciclos microbianos que operan en el sistema. Para ello, se aplicaron técnicas independientes de cultivo basados en la subunidad pequeña del RNA ribosomal (SSU RNA), como electroforesis en gel de gradiente desnaturalizante (DGGE), secuenciación del gen de 16S rRNA (clonaje) y la variación de la hibridación *in situ* con sondas fluorescente con deposición catalizada (CARD-FISH). Microorganismos relacionados con el ciclo del hierro (*At. ferrooxidans*, *Sulfobacillus* spp., *Ferroplasma* spp., etc.), azufre (*Desulfurella* spp., *Desulfosporosinus* spp., *Thermodesulfobium* spp., etc.), carbono (*Acidiphilium* spp., *Bacillus* spp., *Clostridium* spp., *Acidobacterium* spp., etc.) y nitrógeno (*Alcaligenes* spp., *Pseudochrobactrum* spp., etc.) fueron identificados y su distribución correlacionada con los parámetros fisicoquímicos de los sedimentos. En aquellos puntos donde el pH y el potencial redox son mas cercanos a la columna de agua (pH 2,5 y +400 mV), los organismos predominantes fueron identificados como bacterias reductoras de hierro: *Acidithiobacillus* spp. y *Acidiphilium* spp., probablemente relacionado con la alta solubilidad del hierro a bajo pH. En cambio, a mayor pH (4,2-6,2) y condiciones redox mas reductoras (50, -210 mV) dominaban bacterias sulfatoreductoras de los generos *Syntrophobacter, Desulfosporosinus* y *Desulfurella.* El diseño de una sonda específica para los reductores de azufre pertenecientes al género *Desulfurella* fue primordial para el trabajo.

Adicionalmente, se han usado cultivos de enriquecimiento específicos para validar este modelo y probar la existencia de las potenciales actividades anaerobias que habían sido inferidas en los sedimentos ácidos del río Tinto. Se obtuvieron resultados positivos para cultivos de metanógenas, sulfatorreductoras, desnitrficantes, reductoras de hierro y productoras de hidrógeno. Algunos microorganismos han podido ser aislados como sulfatorreductoras acidófilas (dos posibles nuevas especies y un posible nuevo género relacionados con *Desulfosporosinus*/*Desulfitobacterium*), fermentadoras (un posible nuevo género, próximo a *Paludibacter*) y productoras de hidrógeno.

Finalmente, se ha ensayado la capacidad de biorremediar aguas ácidas de mina, usando los sedimentos del río Tinto como fuente de bacterias sulfatorreductoras, y aguas residuales urbanas como fuente de carbón económica. Se obtuvo un efluente libre de metales pesados.

ABSTRACT

Tinto river (Huelva, Spain) is a natural acidic rock drainage (ARD) environment produced by the bio-oxidation of metallic sulfides from the Iberian Pyritic Belt. So far, and despite their ecological interest, the underlying sediments were studied only very sparsely and no complete studies were undertaken. In this thesis, an extensive survey of the Tinto River anaerobic sediment microbiota is presented combining culture independent and dependent methods following the "full-cycle rRNA approach".

A geomicrobiological model of the different microbial cycles operating in the sediments has been developed through molecular biological methods. Culture independent methods targeting the small subunit ribosomal RNA (SSU rRNA) such as denaturing gradient gel electrophoresis (DGGE), 16S rRNA gene sequencing (cloning) and catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) were used. Microorganisms involved in the iron (*Acidithiobacillus ferrooxidans*, *Sulfobacillus* spp., etc.), sulfur (*Desulfurella* spp., *Desulfosporosinus* spp., *Thermodesulfobium* spp., etc.), carbon (*Acidiphilium* spp., *Bacillus* spp., *Clostridium* spp., *Acidobacterium* spp., etc.) and nitrogen (*Alcaligenes* spp., *Pseudochrobactrum* spp., etc.) cycles were identified and their distribution correlated with physicochemical parameters of the sediments. Where the pH and redox potential are closer to those of the water column (pH 2.5 and +400 mV), the most abundant organisms were identified as iron-reducing bacteria: *Acidithiobacillus* spp. and *Acidiphilium* spp., probably related to the higher iron solubility at low pH. At higher pH (4.2-6.2) and more reducing redox potential (50, -210 mV) and therefore, lower solubility of iron, members of the sulfate-reducing genera *Syntrophobacter, Desulfosporosinus* and *Desulfurella* were dominant. The design of a specific probe (DSU655) targeting sulfur-reducing organisms belonging to *Desulfurella* genus was conclusive to the work.

Additionally, we used targeted enrichment incubations to validate this model and prove the existence of the potential anaerobic activities detected in the acidic sediments of Tinto River. Methanogenic, sulfate-reducing, denitrifying, iron-reducing and hydrogen-producing enrichments yield positive results. Classical techniques for bacterial isolation were applied and some microorganisms were isolated such as acidophilic sulfate-reducing bacteria (two new species and a new genus, related to the *Desulfosporosinus*/*Desulfitobacterium* cluster), fermenters (a new genus, closed related to *Paludibacter*) and hydrogen-producers.

Finally, a biotechnological application using Tinto river sediments was tested. Bioremediation of acid rock drainage (ARD) with sulfate-reducing bacteria using domestic wastewater (DW) as a cost-effective carbon-source showed a complete cleaning of the ARD water obtaining an effluent with neutral pH and no metal content.

SUMMARY OF CHAPTERS

In Chapter 1 an extensive survey of the Tinto River sediment microbiota using two culture independent approaches: denaturing gel gradient electrophoresis (DGGE) and cloning of 16S rRNA genes in two physic-chemically contrasting sampling sites (*SN* and *JL* dams) is presented. The taxonomic affiliation of the *Bacteria* showed a high degree of biodiversity, falling into five different phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Acidobacteria* and *Actinobacteria*, while all the *Archaea* were affiliated to the *Thermoplasmatales* group. Microorganisms involved in the iron (*At. ferrooxidans*, *Sulfobacillus* spp., *Ferroplasma* spp., etc.), sulfur (*Desulfurella* spp., *Desulfosporosinus* spp., *Thermodesulfobium* spp., etc.) and carbon (*Acidiphilium* spp., *Bacillus* spp., *Clostridium* spp., *Acidobacterium* spp., etc.) cycles were identified and their distribution correlated with physicochemical parameters of the sediments. Ferric iron was the main electron acceptor for the oxidation of organic matter in the most acid and oxidizing layers, so acidophilic facultative Fe(III)-reducing bacteria appeared extensively in the clones libraries. With increasing pH, the solubility of iron decreases and sulfate-reducing bacteria become dominant, the ecological role of methanogens being scarce. Considering the identified microorganisms – which according to the rarefaction curves and Good´s coverage values cover

almost all of the diversity - and their corresponding metabolism, a model of the iron, sulfur and carbon cycles in an ARD-related sediments is proposed.

In Chapter 2, the abundance of diverse microbial populations inhabiting both physic-chemically contrasting sampling sites (*SN* and *JL* dams) was quantified. Depth profiles of total cell numbers differed greatly between the two sites, yet were consistent in decreasing sharply at greater depths. Although catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) with domain-specific probes showed that *Bacteria* (>98%) dominated over *Archaea* (<2%) at both sites, important differences were detected at the class and genus level reflecting differences in pH, redox potential and heavy metal concentrations. At *SN*, where the pH and redox potential are similar to those of the water column (pH 2.5 and +400 mV), the most abundant organisms were identified as iron-reducing bacteria: *Acidithiobacillus* spp. and *Acidiphilium* spp., probably related to the higher iron solubility at low pH. At the *JL* dam, characterized by banded sediment with higher pHs (4.2-6.2), more reducing redox potential (50, -210 mV) and a lower solubility of iron, members of sulfate-reducing genera *Syntrophobacter, Desulfosporosinus* and *Desulfurella* were dominant. The latter was quantified with a newly designed CARD-FISH probe (DSU655). In layers where sulfate-reducing bacteria were abundant, pH was higher and redox potential, dissolved metals and iron were lower. These results suggest that the attenuation of ARD characteristics is biologically driven by sulfate-reducers and the consequent precipitation of metals and iron as sulfides.

In Chapter 3, a combination of molecular biological methods and targeted enrichment incubations was used to validate the formerly proposed model and prove the existence of the inferred potential anaerobic activities in the acidic sediments of Tinto River. Methanogenic, sulfate-reducing, denitrifying and hydrogen-producing enrichments were all positive at pH between 5 and 7. Methanogenic enrichments revealed the presence of methanogenic archaea belonging to the genera *Methanosarcina* and *Methanobrevibacter*. Enrichments for sulfate-reducing microorganisms were dominated by *Desulfotomaculum* spp. Denitrifying enrichments showed a broad diversity of bacteria belonging to the genera *Paenibacillus*, *Bacillus*, *Sedimentibacter*, *Lysinobacillus*, *Delftia*, *Alcaligenes*, *Clostridium* and *Desulfitobacterium*. Hydrogen-producing enrichments were dominated by *Clostridium* spp. These enrichments confirm the presence of anaerobic activities in the acidic sediments of the Tinto river that are normally assumed to take place exclusively at neutral pH.

In Chapter 4, we complete the overview of the microbial diversity of the sediments -increasing the cloning effort and the studied sites- and combine it with culture-dependent methods -enrichments for iron-reduction, methanogenesis, denitrification and sulfate-reduction- to pool all the latter studies in a comprehensive common discussion at a global scale. By cloning, it was possible to phylogenetically identify down to the genus level most of the bacterial sequences. 61 genera were identified, falling in thirteen different phyla: *Synergistetes*, *Cyanobacteria/Chloroplast*, *TM7*, *Chloroflexi*, *Nitrospira*, *Verrucomicrobia*, *Fibrobacter*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, *Bacteroidetes* and *Chloroflexi*. Positive enrichments were obtained for the tested activities in most of the sample sites showing the diversity of metabolisms present along the river. Enrichments at different pH showed that activities such as methanogenesis, denitrification and sulfate-reduction were favored at pHs near 5 while iron-reduction was promoted at lower pHs (~4.5). In the sulfate-reducing enrichment organism belonging to genera *Clostridium* and *Desulfosporosinus* (*Firmicutes*) were

identified. Bacteria sequences from iron-reduction enrichments clustered in the family *Bradyrhizobiacea* (*Alphaproteobacteria)* and *Clostridium* genus. Sequences retrieved from denitrifying enrichments clustered in genera *Alicyclobacillus* and *Desulfurella*. Archaeal sequences of the methanogenic enrichment shared high similarity with *Methanosaeta thermophile*. Remarkably *Methanosaeta concilli* was identified in the sediment used as inocula.

In Chapter 5, sulfate reduction was studied with three sediment samples from Tinto River basin at pHs 4 to 6 with succinate, glycerol, methanol, lactate or hydrogen as electron donors. Stable enrichments of SRB were obtained at a pH as low as 4 with glycerol, methanol and hydrogen as substrates, at pH 4.5 with lactate and at pH 5.5 with succinate. Cloning and sequencing of the 16S rRNA genes showed that fermentative bacteria (*Paludibacter* spp., *Pseudomonas* spp., *Oscillibacter* spp., *Variovorax* spp.) and SRB (*Thermodesulfobium* spp, *Desulfosporosinus* spp., *Desulfitobacterium* spp., *Desulfotomaculum* spp.) were co-enriched. By repeated serial dilutions and streaking on agar plates, 4 strains of SRB were isolated. For three of the isolates, the highest 16S rRNA gene similarity with characterized species is 96%. Two of them are closely related to *Desulfosporosinus acidophilus* and one is closely related to *Desulfosporosinus orientis*. One isolate that has just 93% rRNA gene sequence similarity with the *Desulfosporosinus*/ *Desulfitobacterium* cluster, might represent a novel species within a novel genus.

In Chapter 6, domestic wastewater (DW) was tested as a cost-effective carbon-source for the bioremediation of acid rock drainage (ARD) with sulfate-reducing bacteria. Sediments from Tinto were used as inoculum. Three anaerobic bioreactors with different microbial supports were operated, fed with a 1:10 mixture of ARD:DW. Around 50% of the organic matter present in the DW co-precipitated with the metals from the AMD previous to feeding the reactor. Therefore, the reactors had to be supplemented with an extra carbon-source (acetate) to achieve high S elimination. Elevated removal efficiencies of COD (>88 %), sulfate (>75 %), Fe_{Tot} (>85 %) and other dissolved metals (>99 % except for Mn) were achieved. Bacterial communities were examined through denaturing gradient gel electrophoresis and scanning electron microscopy. Higher biodiversity was found in the bioreactors compared to that of the inoculum. Dominant species belong to two metabolic groups: fermentative (*Clostridium* spp., *Paludibacter* spp. and *Pelotomaculum* spp.) and sulfate-reducing bacteria (*Desulfomonile* spp., *Desulfovibrio* spp., *Desulfosporosinus* spp., *Desulfurella* spp. and *Desulfotomaculum* spp).

INTRODUCCIÓN

GEOQUÍMICA DEL RÍO TINTO

La **Faja Pirítica Ibérica (FPI)**, inmersa en la zona geotectónica sud-portuguesa de la Península Ibérica, presenta unas dimensiones de más de 250 km de largo y 20-75 km de ancho (Fig. 1). Está constituida mayoritariamente por depósitos minerales de sulfuros de hierro y cobre, y en menor cantidad de plomo y zinc (39).

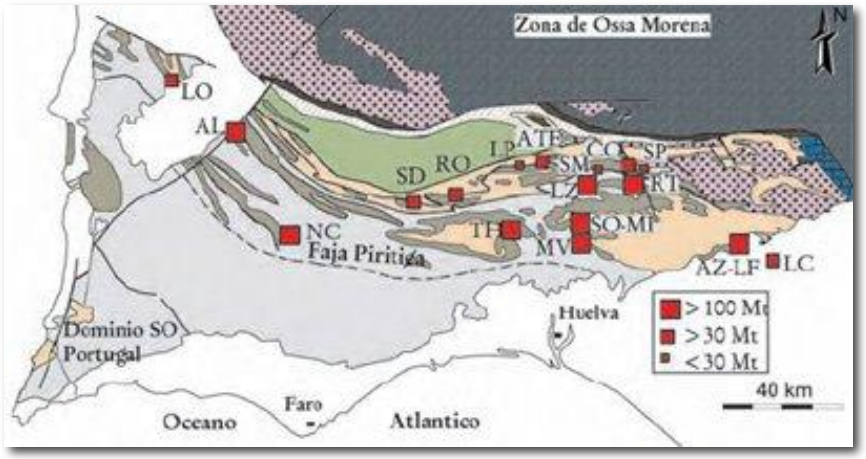

Figura 1: Mapa geológico del área de río Tinto (72).

El distrito minero de río Tinto es uno de los ocho grandes depósitos de sulfuros masivos de la Faja Pirítica Ibérica y quizás la mayor concentración de sulfuros masivos en la corteza terrestre, con más de 400 Mt de sulfuros masivos y unos 2000 Mt de reservas de baja ley (45). Esta formación de origen volcánico se depositó en un ambiente hidrotermal oceánico durante la orogenia Hercínica. Posteriormente, durante una etapa del Terciario tardío, se elevó quedando expuesta a la erosión (9, 18, 39).

Cuando estos sulfuros son expuestos al aire o agua, su **oxidación** puede llevarse a cabo con oxígeno atmosférico o más bien con hierro férrico, cuya tasa de oxidación es de 18 a 170 veces mayor que la del oxígeno. Dependiendo de la estructura cristalina del mineral, los sulfuros pueden ser oxidados por distintas vías (60).

La pirita (FeS_2), molibdenita (MoS_2) y tungstenita (WS_2) son oxidadas siguiendo la vía del tiosulfato. El ión férrico en condiciones de baja presión y temperatura, rompe los enlaces entre el hierro y el disulfuro en la red cristalina del mineral, oxidando a éste último parcialmente a tiosulfato (ec. 1) que será posteriormente oxidado completamente por el hierro férrico hasta sulfato (ec. 2). En esta vía, el principal producto de la reacción global es el ácido sulfúrico (67).

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+ \qquad \text{(ec. 1)}$$
$$S_2O_3^{2-} + 8Fe^{3+} + 5H_2O \rightarrow 2SO_4^{2-} + 8Fe^{2+} + 10H^+ \qquad \text{(ec. 2)}$$

El resto de los sulfuros como la calcopirita ($CuFeS_2$), esfalerita (ZnS), galena (PbS), etc. son oxidados a través de la vía de los polisulfuros con un ataque combinado de hierro férrico y protones. En esta vía, se generan polisulfuros (ec. 3) que se oxidan parcialmente a azufre elemental (ec.4).

$$8MS + 8Fe^{3+} + 8H^+ \rightarrow 8M^{2+} + 4H_2S_n + 8Fe^{2+} \quad (n \geq 2) \quad \text{(ec. 3)}$$
$$4H_2S_n + 8Fe^{3+} \rightarrow S_8^{\circ} + 8Fe^{2+} + 8H^+ \qquad \text{(ec. 4)}$$

Para la oxidación completa de estos sulfuros, se requiere el concurso de microorganismos oxidadores de azufre (*Acidithiobacillus ferrooxidans*, *At. thiooxidans*, etc.), capaces de oxidar el azufre elemental a ácido sulfúrico según la reacción 5:

$$S_8° + 12O_2 + 8H_2O \rightarrow 8SO_4^{-} + 16H^{+} \text{ (ec. 5)}$$

En resumen, la completa oxidación de los sulfuros por la vía del tiosulfato está bajo control estrictamente electroquímico mientras que por la vía de los polisulfuros se requiere la acción de oxidadores de azufre para oxidar el azufre completamente hasta sulfato. Sin embargo, en ambas vías, los microorganismos acidófilos quimiolitótrofos juegan un papel clave. *Leptospirillum* spp. o *Acidithiobacillus* spp. contribuyen al proceso aumentando hasta 5 ordenes de magnitud la tasa de oxidación del ión ferroso (ec. 6), manteniendo así una alta concentración de ión férrico, el agente oxidante responsable del proceso (60).

$$4Fe^{2+} + O_2 + 2H^{+} \rightarrow 2Fe^{3+} + 2H_2O \qquad \text{(ec. 6)}$$

El **río Tinto** nace en Peña de Hierro, en las cercanías del Pico del Padre Caro, en el corazón de la FPI, a una altura de 701 metros sobre el nivel del mar recorriendo 92 km antes de desembocar en el Océano Atlántico, en la ciudad de Huelva. La cuenca del río Tinto atraviesa la provincia de Huelva de noreste a sudoeste cubriendo una extensión de 1.676 km^2 con un suave desnivel del 6%. De norte a sur, el río atraviesa los municipios onubenses de Nerva, Minas de Río Tinto, El Campillo, Zalamea la Real, Berrocal, Valverde del Camino, Paterna del Campo, Niebla, La Palma del Condado, Villarrasa, Bonares, Trigueros, Lucena del Puerto, San Juan del Puerto, Moguer, Palos de la Frontera y Huelva.

Los productos de la oxidación de los minerales que sirven de lecho al río (hierro férrico y ácido sulfúrico) son los responsables de sus peculiares características. En primer lugar, su nombre - Tinto - refleja el intenso color rojo de sus aguas (Fig. 2), causado por la elevada concentración de hierro férrico en solución (~2 g L^{-1}).

Figura 2: Imagen del río Tinto y el intenso color rojo de sus aguas.

El ión férrico también otorga un potencial redox altamente oxidante (~400 mV). El ácido sulfúrico producido en la oxidación es responsable de la alta concentración de sulfatos en sus aguas (~6 g L^{-1}) y de la extrema y constante acidez (pH~2,3) que está, a su vez, tamponada por la reacción de *hidrólisis* del férrico (ec. 7). El ión férrico en presencia de agua precipita como hidróxido férrico y libera protones, lo que mantiene constante el pH del agua al compensar con su equilibrio químico las fluctuaciones en el caudal del río (42).

$$Fe^{3+} + 3H_2O \leftrightarrow Fe(OH)_3 + 3H^+ \quad \text{(ec. 7)}$$

Cuando estas aguas entran en contacto con las vetas de sulfuros metálicos, el bajo pH y el ión férrico facilitan la solubilidad de los metales, lo que explica las altas concentraciones de metales pesados en disolución (Cu: ~0,1 g L^{-1}; Zn: ~0,2 g L^{-1}, etc.) (33).

Las condiciones geoquímicas del río definen tres **zonas principales** (63). En primer lugar la zona norte (desde Peña de Hierro a La Palma del Condado), consistente en un complejo volcánico paleozoico caracterizado por altas concentraciones de férrico en solución y un pH constante de 2.3. En segundo lugar, la zona de transición (desde La Palma del Condado a San Juan del Puerto) con un substrato mixto compuesto por rocas paleozoicas, arcillas terciarias y terrazas fluviales cuaternarias que muestran un ligero incremento del pH hasta 3. Por último, el estuario (desde San Juan del Puerto al océano Atlántico), la sección con influencia de mareal, caracterizada por su menor contenido en hierro y su mezcla con aguas salinas de mayor pH (9). De acuerdo con los parámetros bioclimáticos, el área norte se corresponde con un clima subhúmedo meso-termomediterráneo, mientras que la zona de transición y el estuario podrían ser incluidas en un clima termomediterráneo subhúmedo/seco (10, 59).

La FPI ha sido sujeta a **actividades mineras** durante miles de años (66). Comenzando en la Edad de Bronce, los Íberos (año 3.000 a. C.) - que denominaban al río Tinto *Iberus* -, tartesos, fenicios, romanos – que lo llamaban *Urium* - y los musulmanes, han explotado sus recursos minerales. En el siglo XIX, comenzó la explotación minera a gran escala, principalmente llevada a cabo por empresas del Reino Unido como *Rio Tinto Company Limited*. Por su antigua tradición minera, se consideraba que el río Tinto era un ambiente producto de la contaminación (38). Sin embargo, se ha probado que las condiciones ácidas del Tinto son más antiguas que las explotaciones mineras al datar las formaciones de hierro de los depósitos sedimentarios de jarosita, coquimbita y copiapita (precipitados necesariamente en condiciones de acidez) en más de 2 millones de años (18, 19).

Por sus peculiares características, el río Tinto ha sido objeto de diversos **campos de investigación**. Por un lado, tiene tanto interés biogeoquímico -ahondando en la naturaleza de las comunidades microbianas asociadas con las aguas ácidas de mina (*Acid Mine Drainage*, AMD)- como puramente microbiológico, ya que los ambientes ácidos ofrecen una oportunidad única para estudiar la complejidad de hábitats biológicos en condiciones extremas. Además, el río Tinto posee un gran potencial biotecnológico ya que los organismos que lo pueblan son usados en biominería (biolixiviación), desulfuración de carbones, etc. Por último, el río Tinto tiene un alto interés astrobiológico: ha sido propuesto como un análogo de Marte ya que ambos ambientes parecen poseer una mineralogía común. Las misiones MER (*Mars Exploration Rover*) de la NASA (8, 18) han descubierto unidades litológicas con alto contenido en hierro y sulfatos sobre la superficie de Marte.

BIODIVERSIDAD DEL RÍO TINTO

Como consecuencia del gran interés suscitado por el río Tinto, se conoce mucho sobre la microbiota que habita sus aguas. El uso combinado de métodos convencionales y de ecología molecular ha llevado a la identificación de los organismos más representativos de la columna de agua de la cuenca del Tinto (1-3, 20, 21, 23, 24, 26, 41, 42, 62, 76).

Aproximadamente el 80% de la biomasa **procariótica** en la columna de agua corresponde a tan sólo tres bacterias: *A. ferrooxidans*, *L. ferrooxidans* y *Acidiphilium* spp., todas ellas relacionadas con el ciclo del hierro. *A. ferrooxidans* es una bacteria acidófila anaerobia facultativa y quimiolitótrofa altamente versátil: es capaz de oxidar hierro y azufre en condiciones aerobias y de reducir hierro oxidando azufre en condiciones anaerobias (44). *L. ferrooxidans* es una bacteria acidófila aerobia estricta y quimiolitótrofa que oxida hierro y parece capaz de fijar N2 (21). Por último, las especies del género *Acidiphilium* son heterótrofas, capaces de usar el oxígeno como aceptor de electrones o bien, el ión férrico en condiciones de microaerobiosis (16). Otros organismos oxidadores de hierro, como las arqueas *Ferroplasma* spp. o *Thermoplasma* spp., o reductores de hierro, como *Ferrimicrobium* spp., también han sido identificados pero su baja abundancia sugiere que juegan un papel minoritario en la ecología del Tinto (23, 25). Aunando toda esta información, se planteó un modelo de funcionamiento de los organismos en la columna de agua (Fig. 3).

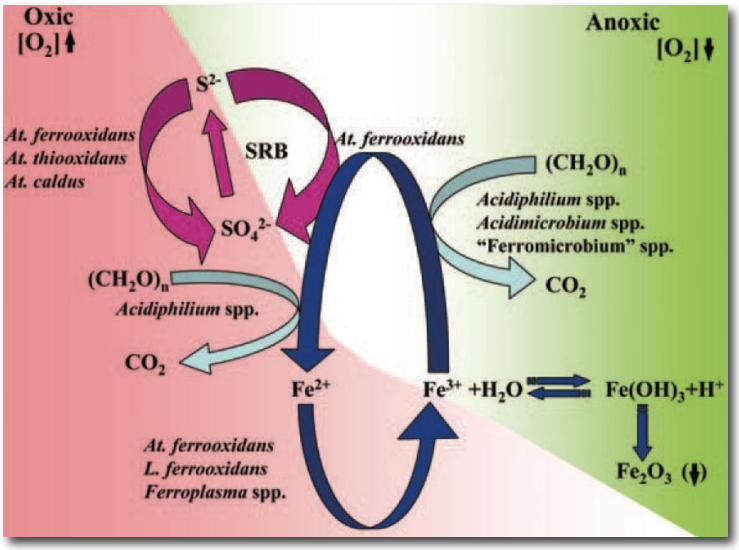

Figura 3: Modelo de la ecología microbiana del río Tinto. Metabolismo del hierro: el ion ferroso sería oxidado por bacterias como *At. ferrooxidans, L. ferrooxidans, Ferrimicrobium* spp., *Acidimicrobium* spp. y por la arquea *Ferroplasma* spp. El ion férrico sería reducido en condiciones de anaerobiosis por *Acidimicrobium* spp., *Ferrimicrobium* spp. y *Acidiphilium* spp., utilizando compuestos reducidos de carbono ($(CH_2O)_n$) como fuente de energía o por *At. ferrooxidans* oxidando compuestos reducidos del azufre. Respecto al ciclo del azufre, bacterias quimiolitoautótrofas como *At. ferrooxidans, At. thiooxidans* y *At. caldus*, serían responsables de oxidar azufre a sulfato. Además, en ausencia de oxígeno *At. ferrooxidans* llevaría a cabo la oxidación de azufre acoplada a la reducción de hierro como se explicó anteriormente. En cuanto a la regeneración del sulfato podría llevarse a cabo por sulfatorreductoras (26).

A lo largo del río aparecen unas estructuras filamentosas macroscópicas de color marrón (Fig. 4), denominadas serpentinas o *streamers*, compuestas por los tres grupos de bacterias mayoritarias de la columna de agua (*Acidithiobacillus*, *Leptospirillum* y *Acidiphilium*) inmersas en una matriz de exopolisacáridos y partículas minerales. Minoritariamente aparecen algunos

microorganismos de metabolismo anaerobio, lo que sugiere que podrían ser originados en los sedimentos y elevarse hasta la superficie al esponjarse y hacerse menos densos (20).

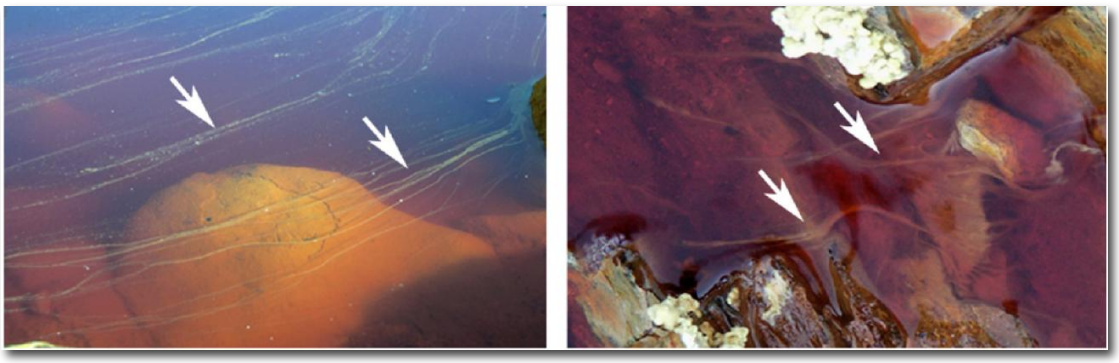

Figura 4: Serpentinas suspendidas en las aguas del río (20).

A pesar de la baja diversidad procariota, y de la creencia generalizada de que los metales pesados inhiben el crecimiento y diversidad de los eucariotas (28), en el río Tinto aparece una inesperada **diversidad eucariótica** (1-3, 76) que incluye algas, ciliados, flagelados, amebas, hongos y rotíferos. Coloridas biopelículas que cubren grandes superficies, comunidades filamentosas y algas macroscópicas son características comunes en las aguas del Tinto.

La mayoría de las especies eucariotas son fotosintéticas siendo los principales contribuyentes a la biomasa (>65%) en este hostil río (42). Estas algas son mayoritariamente clorofitas (géneros *Chlamydomonas*, *Chlorella*, *Euglena, Dunaliella,* etc.) y aparecen formando largos filamentos verdes (Fig. 5) (1-3). En las zonas con características más extremas, predomina el género de rodofita *Cyanidium* con la clorofita *Dunaliella,* uno de los organismos más extremos descritos hasta ahora (22). También aparecen, especialmente durante los meses secos, algas filamentosas de los géneros *Zygnemopsis* y *Klebsormidium*. Diatomeas penadas del género *Pinnularia* (y en ocasiones *Nitzchia* y *Cyclotella*.) aparecen en el río formando largas biopelículas marrones.

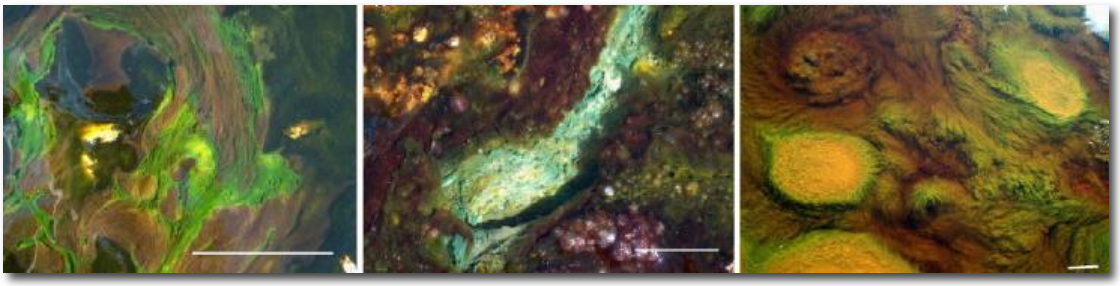

Figura 5: Biopelículas de eucariotas en río Tinto. A) Euglenas y diatomeas, B) *Cyanidium* y diatomeas, C) *Euglena*, *Chlamydomonas* y hongos (3).

Los protistas heterótrofos también están ampliamente distribuidos a lo largo del río. Flagelados mixotrofos del género *Bodo* y *Ochromonas* y ciliados del género *Oxytrichia* han sido identificados. Frecuentemente aparecen amebas, tanto del tipo lobosea como acantameba, que se alimentan de las diatomeas. También han sido identificados heliozoos del género *Actiniphyris*. Entre los descomponedores, los hongos son los más abundantes y aparecen tanto levaduras como hongos filamentosos (41, 42). Los hongos parecen tener un papel fundamental en la formación de las biopelículas y aparecen asociados a las principales bacterias de la columna de agua (70). El único animal encontrado en el río es una especie de rotífero bdeloide

relacionado con el género *Rotifera* (76) que parece subsistir gracias a su alta tolerancia al estrés ácido y a la ausencia de competidores más eficientes.

Los **sedimentos** del Tinto poseen un gran interés ecológico (permiten cerrar los ciclos biogeoquímicos de los ambientes ácidos de mina), biotecnológico (organismos con aplicaciones a bio-pilas, recuperación de metales, inmovilización de radionucleidos, …) y astrobiológico (la atmósfera de Marte carece de oxígeno por lo que los metabolismos que pudieran encontrarse serían anaerobios). A pesar de ello y por las limitaciones metodológicas de su estudio, hasta ahora había un conocimiento muy limitado de su microbiota. En río Tinto, sólo había sido descrita la presencia de arqueas metanogénicas (68) y alguna especie sulfato-reductora (20), pero sin llegar a valorar su diversidad y funcionamiento de manera profunda. Por otro lado, en perforaciones rocosas, el denominado proyecto MARTE (7), se detectó la existencia de actividades anaerobias a altas profundidades y la presencia de gas metano, oxido nítrico e hidrógeno.

Estos antecedentes sugerían la posibilidad de existencia y diversidad de actividades anaerobias en el subsuelo del río Tinto y abrían la cuestión de cómo sería la ecología microbiana de los sedimentos, qué organismos estarían presentes, su abundancia y cuáles serían las rutas metabólicas presentes en los sedimentos de este hostil ecosistema. Este ha sido el ambicioso objetivo de la presente tesis.

POTENCIALES PROCESOS MICROBIANOS EN LOS SEDIMENTOS DEL RÍO TINTO.

Los parámetros ambientales de un ecosistema, tales como temperatura, concentración de oxígeno, flujos de nutrientes, pH, características geológicas, etc., influyen en propiedades de los sedimentos tales como el tamaño de partícula, el contenido de materia orgánica, la tasa de sedimentación, etc. En el río Tinto encontramos sedimentos con distinta granulometría. A lo largo de su cuenca mayoritariamente se producen acumulaciones de partículas minerales de cierto tamaño - arenas y grava - con alta porosidad y sin estructura real de sedimento. En ellas, el principal mecanismo de transporte será la convección y sus características biogeoquímicas estarán fuertemente influenciadas por las de la columna de agua. Sin embargo, existen ciertas zonas con presas o con menor pendiente donde se reduce la velocidad lineal de la masa de agua permitiendo una acumulación estratificada de sedimentos con menor tamaño de partícula, principalmente limos. En ellos el principal mecanismo de transporte será la difusión, lo que crea mayor variedad de posibles nichos ecológicos con características que pueden diferir de las del agua superficial. En los sedimentos esta variabilidad se refleja en una diversidad de comunidades microbianas y procesos biogeoquímicos (31, 40, 61).

Los procesos biológicos que se dan en un sistema dependen de la energética de las reacciones, favoreciéndose aquellos que obtienen mayor energía por mol de substrato. La fuente de energía para el mantenimiento celular puede ser química (quimiótrofos) o lumínica (fotótrofos). Los organismos fotótrofos convierten la energía lumínica en energía metabólica y poder reductor pero en los sedimentos del río Tinto, debido a la ausencia de luz, predominarán los metabolismos basados en la quimiotrofía (quimio-organotrofía o quimio-litotrofía). A su vez, la fuente de carbono puede ser inorgánica (autótrofos) u orgánica (heterótrofos).

Hay dos tipos de metabolismos quimiótrofos principales: la fermentación y la respiración. En la **fermentación** no hay un aceptor externo de electrones por lo que no requieren de una cadena

transportadora y no hay cambio global del estado de oxidación. Como consecuencia, el rendimiento energético es bajo (2-3 ATP por mol hexosa). Hay distintos tipos de fermentación: alcohólica, láctica (homo o heteroláctica), propiónica, ácido-mixta, acetona-butanol que generan como productos distintos tipos de ácidos grasos volátiles, alcoholes e hidrógeno.

En los **procesos de respiración**, el dador primario de electrones cede los electrones al NAD^+ reduciéndolo a $NADH,H^+$ desde el que fluyen a través de la cadena transportadora de electrones que irá traslocándolos -a través de citocromos y quinonas- hasta el aceptor final de electrones, generando en el proceso un gradiente de protones a ambos lados de la membrana usado para realizar un trabajo: químico (almacenar energía en forma de ATP), cinético (movimiento celular), de transporte, etc. De un modo resumido, la energía obtenida en este proceso será mayor cuanto mayor sea la diferencia de potenciales de reducción de los elementos que intervengan (Fig. 6), es decir, la energía libre de Gibbs (ΔG^o) (ec. 8) es proporcional a la diferencia del potencial de reducción entre el aceptor de electrones (oxidante, elemento que se reduce) y el dador de electrones (reductor, elemento que se oxida).

$$\Delta G^{o\prime} = -n \cdot F \cdot \Delta\varepsilon = -n \cdot F \cdot \Delta\varepsilon = -n \cdot F \cdot (\varepsilon_{REDUCCIÓN}{}^o - \varepsilon_{OXIDACIÓN}{}^o) \quad \text{(ec. 8)}$$

siendo F la constante de Faraday, cuyo valor es de 23 kcal mol^{-1}.

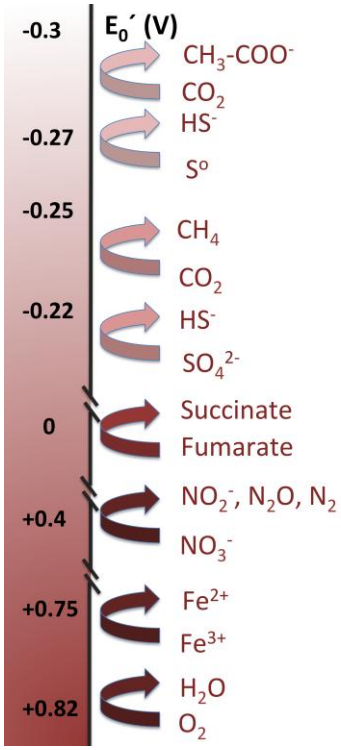

Figura 6: Torre de potenciales. Los pares redox se expresan como compuesto oxidado/reducido (ej. O_2/H_2O). Cuanto mayor, más positivo sea el potencial de reducción de un par redox, más tendencia tendrá a reducirse, esto es a actuar como aceptor de electrones.

De modo que, ante cierta disponibilidad de diferentes dadores y aceptores de electrones, la secuencia de aceptores preferenciales de electrones (Fig. 6) sería: oxígeno (respiración aerobia), hierro (reducción de hierro), nitrato (desnitrificación), sulfato (sulfato-reducción) y dióxido de carbono (metanogénesis y, posteriormente, acetogénesis). Puesto que la energía libre liberada depende no sólo de los potenciales estándar, si no también de las concentraciones de dador y aceptor de electrones (ec. 9), el orden puede alterarse. Esto es

especialmente significativo en el caso de la reducción de hierro puesto que dada su baja solubilidad resulta menos eficiente que la desnitrificación.

$$\Delta G^{\circ\prime} = -n \cdot F \cdot \Delta\varepsilon + \log [\text{forma oxidada}] / [\text{forma reducida}] \qquad \text{(ec. 9)}$$

En las primeras capas de sedimentos se dan los procesos de **respiración aerobia**, los más favorables energéticamente (ej. 34 ATP por mol de hexosa), oxidándose la materia orgánica presente y/o los compuestos reducidos de los minerales hasta CO2 o las formas oxidadas de las moléculas minerales. Su rápido consumo hace que generalmente el oxígeno disponible desaparezca en los primeros milímetros (40).

Cuando la concentración de oxígeno se reduce por debajo de 2-8 µM, el nitrato se convierte en el mejor aceptor de electrones (13). La **desnitrificación** puede llevarse a cabo tanto por bacterias heterótrofas como autótrofas. En la desnitrificación heterótrofa, un sustrato orgánico, como metanol, etanol, ácido acético, glucosa, etc., actúa como fuente de energía (dador de electrones) y fuente de carbono. En la desnitrificación autótrofa, la fuente de energía es inorgánica, como hidrógeno o compuestos reducidos de azufre: sulfhídrico (H_2S), azufre (S°), tiosulfato ($S_2O_3^{2-}$) etc., siendo el CO_2 la fuente de carbono. La capacidad desnitrificante está ampliamente distribuida en bacterias, arqueas y hongos. Los géneros de bacterias desnitrificantes más citados se incluyen: *Alcaligenes*, *Paracoccus*, *Pseudomonas*, *Thiobacillus*, *Rhizobium*, *Thiosphaera*, entre otros. El proceso de reducción de nitratos hasta nitrógeno gas ocurre en etapas seriadas (ecuación 10), catalizadas por sistemas enzimáticos diferentes, apareciendo como productos intermedios nitritos, óxido nítrico y óxido nitroso.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad \text{(ec. 10)}$$

Posteriormente, los aceptores preferenciales serían el manganeso y el hierro. Al contrario del resto de procesos, la **reducción de hierro (Fe^{3+}) y manganeso (Mn^{4+})** es posible en sustratos insolubles, como minerales (13), al conseguir una transferencia de electrones extracelular hasta el aceptor final externo, ya sea por contacto directo (52), quelantes de hierro (43), *pilis* (58). La reducción de hierro ha sido descrita como una de las principales formas de degradación de materia orgánica en sedimentos y los organismos que la llevan a cabo han sido utilizados en biorremediación y producción de energía (74). Pertenecen a diversos grupos filogenéticos siendo especialmente estudiados *Geobacter* y *Shewanella*, por sus aplicaciones en biopilas, y *Acidithiobacillus* por su papel en biolixiviación.

El siguiente proceso energéticamente favorable sería la **sulfato-reducción**. Los organismos sulfatorrectores se definen como organismos anaerobios estrictos, que viven a pH neutro, con la habilidad de llevar a cabo la reducción disasimilativa de sulfato (reducción de sulfato a sulfuro de hidrógeno) obteniendo en el proceso energía para su mantenimiento y crecimiento (ecs. 11 y 12)). Las sulfatoreductoras son un grupo diverso de procariotas (14) divididos en 4 grupos basados en el análisis de secuencias de rRNA: i) bacterias gram-negativas mesófilas (*Desulfobulbus*, *Desulfovibrio*, *Desulfobacter…*), pertenecientes a la clase *Deltaproteobacteria*; ii) bacterias gram-positivas formadoras de endoesporas (*Desulfotomaculum*, *Desulfosporosinus…*), incluidas en la clase *Clostridia*; iii) bacterias termófilas (*Thermodesulfobacterium*), que forman un *phylum* propio; y iv) arqueas termófilas (*Archaeoglobus*), clase *Archaeoglobi*. El sulfato es uno de los aceptores de electrones (junto con el ión férrico) más abundantes en río Tinto, pero dada la poca energía obtenida en el proceso este metabolismo está limitado a zonas completamente anaerobias, donde otros

aceptores más favorables hayan sido consumidos. Sin embargo, se estima que puede llegar a contribuir a la mineralización del 50% de la materia orgánica en los sedimentos (35).

$4\ H_2 + SO_4^{2-} + 2H^+ \text{---> } H_2S + 4\ H_2O$ $\quad \Delta G_0 = -153{,}5\ kJ\ mol^{-1}$ (ec. 11)

$CH_3\text{-}COO^- + SO_4^{2-} + H^+ \text{---> } H_2S + HCO_3^-$ $\quad \Delta G^o = -47{,}1\ kJ\ mol^{-1}$ (ec. 12)

Por último, el paso final en la mineralización de la materia orgánica en condiciones anaerobias es la **metanogénesis**. Ciertos grupos de arqueas utilizan el CO2 como aceptor final de electrones reduciéndolo a metano con hidrógeno. Se denomina metanogénesis hidrogenotrófica (ec. 13) y es llevada a cabo por géneros como *Methanobacterium*, *Methanococcus* o *Methanospirillu*m. Tan sólo dos géneros, *Methanosarcina* y *Methanosaeta,* utilizan el acetato para generar metano en la llamada metanogénesis acetoclástica (ec. 14).

$CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O$ $\quad \Delta G^{o\prime} = -135\ kJ\ mol^{-1}$ (ec. 13)

$CH_3COOH \rightarrow CH_4 + CO_2$ $\quad \Delta G^{o\prime} = -28.4\ kJ\ mol^{-1}$ (ec. 14)

A pesar de que la metanogénesis hidrogenotrófica es más eficiente (ec. 13), energéticamente hablando, que la acetoclástica (ec. 14), el 98% del CO_2 utilizado se emplea para obtener energía y tan sólo el 2% para formar biomasa. A las presiones parciales en las que normalmente se encuentra el hidrógeno en el interior celular, la variación de energía libre se sitúa entorno a 14 kJ mol^{-1} metano generado, lo que permite obtener tan sólo un mol de ATP por mol de metano producido. Ello hace que las arqueas metanogénicas tengan tiempos de generación muy largos, siendo muy sensibles a las variaciones medioambientales. Además, en ambientes con elevadas concentraciones de sulfato, como son los sedimentos marinos o el caso del río Tinto, las bacterias sulfato-reductoras se ven favorecidas sobre las arqueas metanogénicas (ecs. 11 y 12 vs. 13 y 14), siendo las primeras los principales mineralizadores terminales de la materia orgánica.

Existen aceptores de electrones alternativos a los aquí citados, tales como los pares SeO_4^{2-}/SeO_3^{2-} (E: +0,48), AsO_4^{3-}/AsO_3^{3-} (E: +0,16), o compuesto clorados alifáticos y aromáticos, como el par Cl-benzoato/benzoato (E: +0,3), pero su importancia ecológica es, en general, escasa.

ECOLOGÍA MOLECULAR APLICADA AL ESTUDIO DE SEDIMENTOS

Las técnicas de microbiología clásica requieren del aislamiento de cultivos puros. El desconocimiento de las propiedades fisiológicas de un organismo o el hecho de que en la naturaleza muchos organismos aparecen en simbiosis o sinergia con otros - creando condiciones prácticamente imposibles de reproducir en el laboratorio - dificultan o imposibilitan su aislamiento, lo que conlleva grandes sesgos en la identificación de la microbiota de un ecosistema. Además, posteriormente son necesarios múltiples y largos ensayos morfológicos, metabólicos, bioquímicos, genéticos, etc., para la identificación de los aislados. Esto se plasma en un bajo porcentaje de organismos cultivados descritos respecto al total que debe existir, estimado en menos de 1% para diversos ambientes (6). En particular, los ambientes anaerobios han presentado considerables dificultades en este aspecto, debido a las bajas tasas de crecimiento y a los estrictos requerimientos de cultivo. Por ello, en el caso de los sedimentos, este porcentaje se reduce hasta 0,25%.

A finales de los 80´ se desarrollaron una serie de técnicas de biología molecular que supusieron una revolución en el campo de la ecología microbiana (6, 55, 75). Estas técnicas se basaron principalmente en dos grandes avances: i) el descubrimiento de que las relaciones

filogenéticas podían ser inferidas por las secuencias de genes como el 16S rRNA (75) y ii) la amplificación selectiva de ácidos nucleicos de muestras ambientales a través de la reacción en cadena de la polimerasa (*Polymerase Chain Reaction*: PCR) (64). Así surgió la ecología molecular (4), siendo posible la caracterización de la diversidad microbiana de un ecosistema a nivel DNA sin necesidad de enriquecimientos o aislados.

Actualmente la taxonomía microbiana está basada en la información contenida en los ácidos nucleicos (ej. 16S rRNA). La elección del rRNA para la identificación de los organismos y el estudio de su filogenia (55, 56) se fundamenta en su universalidad y abundancia en todos los seres vivos. Al ser parte de la maquinaria para sintetizar proteínas, el RNA ribosomal es una molécula muy preservada. Además, posee un tamaño que lo hace estadísticamente adecuado y fácil de secuenciar con las técnicas actuales. Pero su más preciada característica es su papel como registro evolutivo: asumiendo que los cambios se producen al azar, y que aumentan con el tiempo de manera linear, las diferencias en la secuencia reflejan la distancia evolutiva, permitiéndonos estudiar las divergencias evolutivas entre los distintos organismos. Además, posee zonas con diferentes grados de conservación, regiones muy conservadas y regiones altamente variables, que permiten distinta especificidad desde dominio hasta especie. Adicionalmente, esta molécula no se ve afectada por transferencia horizontal de genes.

A pesar de las innegables ventajas de las técnicas de biología molecular, éstas no permiten obtener información sobre el metabolismo de los taxones identificados. Por lo tanto, es recomendable combinar técnicas de microbiología clásica con técnicas moleculares para conseguir una completa comprensión de un ecosistema. Así este trabajo se ha llevado a cabo basándose en el llamado ciclo completo del rRNA (6, 50, 53) complementando fuertemente la microbiología clásica (Fig. 7).

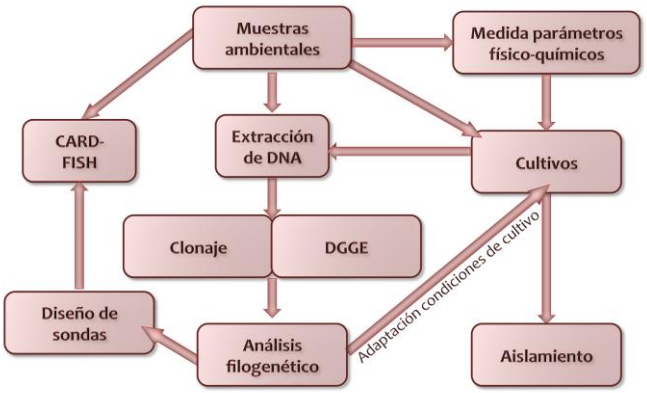

Figura 7: Diagrama de los pasos en el estudio de la estructura y función de las comunidades microbianas. Modificado de (50).

De las técnicas disponibles de ecología molecular, en este trabajo se han aplicado las más empleadas basadas en el rRNA: PCR/DGGE, PCR/clonaje del 16S rRNA y CARD-FISH. Partiendo de una muestra ambiental, y tras medir los parámetros ambientales *in situ*, puede realizarse la extracción ácidos nucleicos como DNA o RNA. Tras ello, puede realizarse un análisis filogenético aplicando técnicas como DGGE y clonaje. El clonaje permite estudios filogenéticos más fiables (54, 73), mientras que la DGGE permite analizar las variaciones temporales o espaciales (50). Con la base de secuencias obtenidas de un ecosistema, pueden aplicarse las sondas complementarias a los organismos presentes o diseñarlas en el caso de que no existan (6, 32, 54), y así cuantificar las distintas poblaciones presentes mediante hibridación *in situ*

(FISH/CARD-FISH). Finalmente, con la información recabada sobre los posibles metabolismos presentes, pueden seleccionarse las condiciones para cultivos de enriquecimiento selectivos de ciertas actividades y, si cabe, proceder al aislamiento de microorganismos. A continuación se explicarán brevemente las técnicas seleccionadas.

ξ **Extracción de DNA**

Tras la toma de muestras, la extracción de DNA de los organismos presentes en el ecosistema es un paso previo necesario a la amplificación por PCR. Hay dos procedimientos generales: i) extracción *in situ*, en el que las células se lisan en la misma matriz de la muestra ambiental, y ii) extracción *ex situ*, donde las células se separan previamente. El primer método es el usado comúnmente por su sencillez, rapidez y cantidad de DNA extraído. Sin embargo, en el caso de sedimentos de un ambiente ácido con alta concentración de metales pesados, este método no es adecuado debido a la co-extracción de ácidos húmicos y metales pesados junto con el DNA (inhibidores de PCR). Adicionalmente, el contenido de células por peso de sedimento es menor en comparación con otros ecosistemas, requiriéndose por tanto técnicas de concentración y purificación previas a la extracción.

ξ **Reacción en cadena de la polimerasa.**

De la compleja mezcla de DNAs que pueden ser extraídas de una muestra ambiental, se pueden amplificar secuencias específicas mediante PCR. La reacción en cadena de la polimerasa se usa para amplificar un determinado segmento de DNA situado entre dos regiones de secuencia conocida. Para iniciar la síntesis de DNA, que será catalizada por la DNA polimerasa, se usan dos cebadores o *primers*: oligonucleótidos complementarios a las secuencias que flanquean el segmento de DNA que se desea amplificar. Primero, para separar las cadenas complementarias de la doble hélice de DNA, se desnaturaliza el DNA molde mediante calor, en una mezcla de reacción con los dos cebadores y los cuatro deoxinucleótidos (dNTPs: dATP, dTTP, dGTP, dCTP). Posteriormente, la mezcla de reacción se enfría hasta una temperatura que permite a los cebadores formar puentes de hidrógeno con sus secuencias diana, tras lo cual la DNA polimerasa comienza a sintetizar DNA a partir de ellos. Este ciclo de desnaturalización, unión de cebadores y síntesis de DNA se repite varias veces, y puesto que los productos de un ciclo sirven de molde para el siguiente, en cada ciclo se dobla la cantidad de DNA. La mezcla de fragmentos amplificados de DNA pude ser usada en posteriores análisis para clonaje y DGGE. Cabe señalar que puede darse la amplificación preferente de algunas secuencias. Además el número de copias de un determinado gen no es el mismo en todos los organismos (por ejemplo, *Mycobacterium tuberculosis* tiene 1 copia, *E. coli* presenta 7 copias y *Bacillus cerus* 12 copias para el gen del 16S rRNA). Por ambas razones esta técnica no puede ser usada de modo cuantitativo para determinar la abundancia natural de los genes amplificados.

Los protocolos originales para la PCR (46, 47, 65) usaban un fragmento de la DNA polimerasa I de *E. coli* para catalizar la síntesis, pero esta enzima se inactiva a las temperaturas requeridas para desnaturalizar el DNA (95ºC), de modo que cada ciclo requería la adicción de nueva enzima. Este problema fue resuelto con la introducción (64) de una DNA polimerasa termoestable, purificada de la bacteria termófila *Thermus aquaticus* (*Taq* DNA polimerasa) (15). Esta enzima no se inactiva al subir la temperatura para desnaturalizar el DNA, por lo que no necesita ser reemplazada en cada ciclo. Actualmente, también se usa otra enzima termoestable: la polimerasa *Vent* de *Thermococcus litoralis*.

ξ Clonaje del gen del 16S rRNA

El clonaje del gen del 16S rRNA para procariotas es una de las técnicas moleculares más utilizada para el estudio de una población microbiana, ya que proporciona de manera fiable información sobre la biodiversidad de un determinado sistema y la relación filogenética de los organismos que lo componen (56).

Generalmente, utilizando cebadores universales del dominio *Bacteria o Archaea,* se procede a la amplificación del gen completo del 16S rRNA contenido en el DNA extraído de una muestra (Fig. 8). Para separar la mezcla de los genes del 16S rRNA de los distintos organismos presentes, éstos son unidos o ligados a un vector de clonación, generalmente un plásmido, que es introducido en un organismo huésped, generalmente células competentes de *E. coli*. Los plásmidos usados contienen un gen de resistencia a la ampicilina y el gen *LacZ* en la zona de inserción del gen a clonar, de tal manera que una vez cultivadas las colonias en placas de agar sólo crecerán las que hayan insertado eficientemente el plásmido. Además, se podrá hacer una selección cromática puesto que la inserción del gen inhibe la expresión del gen *lacZ* produciendo colonias blancas con el inserto y colonias azules sin inserto. La amplificación o extracción de los plásmidos y la posterior secuenciación del 16S rRNA dará como resultado la construcción de una librería de los genes 16S rRNA de los organismos que componen el sistema a estudiar. Las secuencias obtenidas pueden ser utilizadas para realizar estudios filogenéticos, para el diseño de sondas necesarias en otras técnicas de ecología molecular como CARD-FISH o para el diseño de cebadores específicos para amplificación de poblaciones concretas (6, 54). La mayor ventaja de esta metodología es que las secuencias obtenidas contienen el gen completo, lo que proporciona alta precisión en los estudios filogenéticos (71).

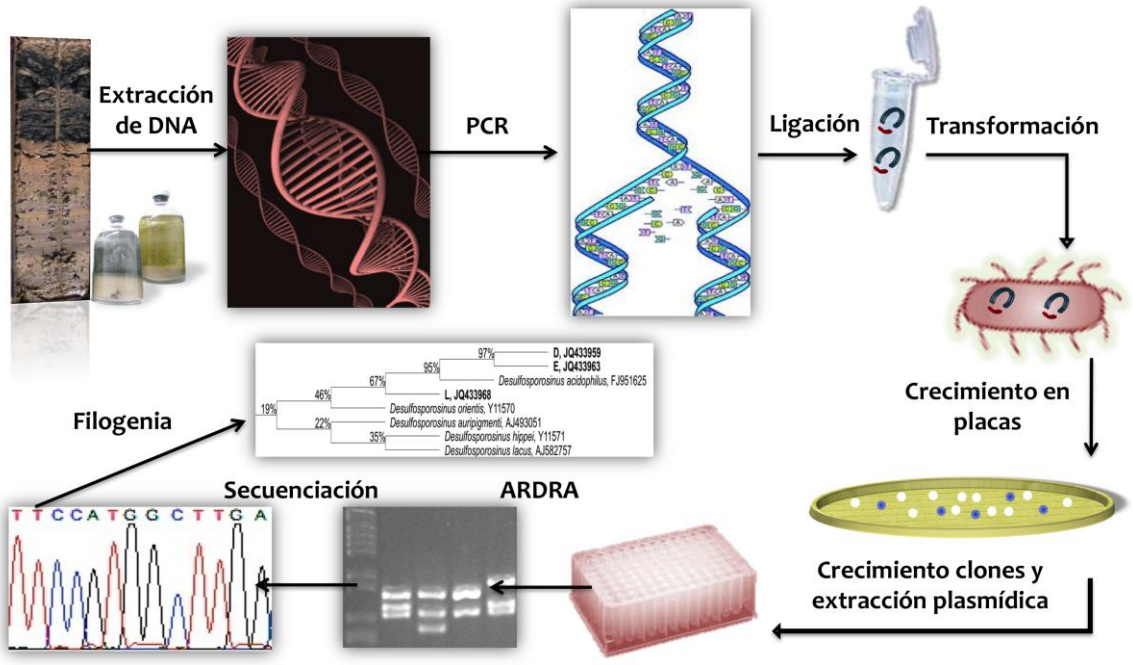

Figura 8: Protocolo de clonaje para el estudio de una comunidad microbiana. Tras la toma de muestra y la extracción de DNA, se lleva a cabo la amplificación del gen completo del 16S rRNA. Distintas copias se insertan en distintos plásmidos (ligación) y después se introducen en células competentes de *E. coli* DH5α (transformación). Tras la selección de las colonias transformadas (blancas) y su crecimiento, se lleva a cabo la extracción del plásmido acorde a sus patrones de restricción. Finalmente tras la secuenciación del gen clonado, se obtiene la afiliación filogenética de las secuencias obtenidas.

ξ **Electroforesis en gel con gradiente desnaturalizante.**

La técnica fue inicialmente desarrollada para el estudio de mutaciones que producen patologías en los genes humanos (12). Su primera aplicación en ecología microbiana fue para el análisis de biopelículas y tapetes microbianos (48). Actualmente es la técnica de huella genética más utilizada en Ecología microbiana, ampliamente aplicada para estudiar los cambios espaciales o temporales en las comunidades en un ecosistema (50).

La DGGE permite la separación de fragmentos de doble hélice de DNA de la misma longitud pero distinta secuencia (49). La técnica se fundamenta en la diferente estabilidad del enlace GC (tres puentes de hidrógeno por enlace) respecto al enlace AT (2 puentes de hidrógeno). Una mezcla de fragmentos de DNA de diferente secuencia se somete a electroforesis a través de un gel de acrilamida con un gradiente químico creciente de sustancias desnaturalizantes (urea-formamida). En general, los fragmentos de DNA ricos en GC serán más estables y mantienen la estructura de doble hélice a mayores concentraciones de desnaturalizantes, hasta que llegan a un punto crítico en el que el DNA se desnaturaliza. Para evitar la separación completa de las dos hebras de DNA, se suele añadir una cola de oligonucleótidos rica en G+C en el extremo del cebador. Ésta cola mantiene unidas las dos hebras complementarias, de tal forma que al liberarse del medio que las mantenía desnaturalizadas, puedan volver a unirse. Este cambio de estructura en el DNA obstruye el movimiento a través del gel: los fragmentos de DNA de doble hélice migran mejor en el gel de acrilamida mientras que los fragmentos desnaturalizados reducen su velocidad y se paran. De este modo, los fragmentos de DNA de diferente secuencia se van separando en el gel de acrilamida. El resultado de un gel de DGGE es un patrón de bandas donde teóricamente cada banda corresponde a un microorganismo diferente (49). La obtención de un patrón de bandas (Fig. 9) es útil para seguir la evolución espacial y temporal de las poblaciones de microorganismos y su respuesta a cambios físico-químicos y nutricionales. Las bandas pueden amplificarse y secuenciarse y generar información taxonómica y filogenética, aunque de menor precisión que con el clonaje (71) dado el menor tamaño de las secuencias (max. 500-600 pb) (27).

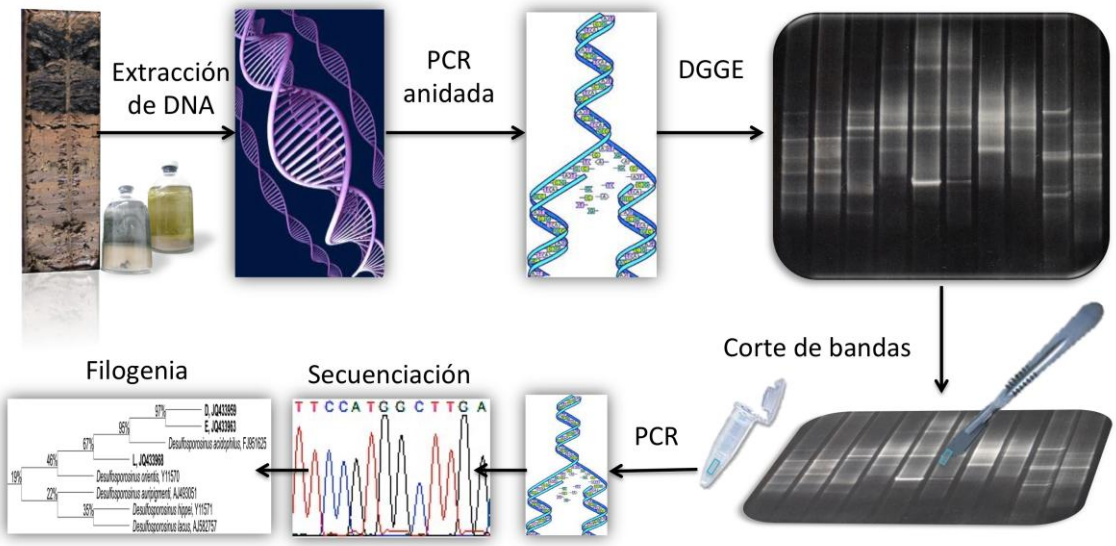

Figura 9: Protocolo de DGGE. Tras la toma de muestra y la extracción de DNA, se lleva a cabo la amplificación de una región del gen 16S rRNA. Los fragmentos generados son sometidos a electroforesis obteniendo un patrón de bandas. Adicionalmente, estas bandas pueden extraerse y tras elución de su DNA, amplificarlo y secuenciarlo.

ξ **Hibridación *in situ* fluorescente**

La técnica de FISH (*Fluorescence In Situ Hybridization)* permite la identificación *in situ* de organismos no cultivados en su ambiente natural, así como la determinación de su abundancia, distribución espacial y morfología celular. Esta técnica fue descrita por primera vez por DeLong y col. en 1989 (17). Se trata de una hibridación DNA-RNA en la una sonda de DNA se une específicamente con una secuencia *firma* del rRNA. Las dianas del 16S rRNA – idóneo por su alto número de copias y su accesibilidad (77) - son secuencias de nucleótidos características de un determinado grupo filogenético, que permanecen invariables dentro de los organismos de un mismo grupo y que no se encuentran en otros. Tras localizarlos, se construye una sonda específica: una pequeña molécula de DNA de unos 18 nucleótidos, marcada en el extremo 5' con un fluorocromo (para su posterior visualización) complementaria a esta región firma del rRNA 16S.

La sensibilidad de esta técnica puede ser problemática si las células no están activas, puesto que la intensidad de la señal depende del número de ribosomas y por tanto de la actividad de las células, o si son demasiado pequeñas y la señal de hibridación está por debajo del limite de detección. En ocasiones, como muestras de sedimentos con autofluorescencia o una gran señal de fondo, la señal de hibridación puede ser difícil de diferenciar del fondo. Para resolver este problema, se han buscado diversos modos de aumentar la intensidad de la señal de fluorescencia a través de fluorocromos más potentes, uso de cloranfenicol, etc. Recientemente se ha desarrollado una modificación del FISH que incrementa notablemente la señal emitida por las moléculas hibridadas, el CARD-FISH, o *Catalyzed Reporter Deposition* (57, 69). La modificación consiste en que la sonda de DNA no va unida directamente al fluorocromo, sino que la sonda va marcada con la peroxidasa de rábano (*HorseRadish Peroxidase*: HRP). En una etapa posterior de amplificación (Fig. 10), se introduce el fluorocromo unido a tiramidas (compuestos fenólicos). La HRP cataliza la unión de las tiramidas a los residuos de tirosina de las proteínas cercanas a la HRP, aumentando notablemente el número de fluorocromos que emiten por ribosoma de la bacteria hibridada: pasa de un fluorocromo por ribosoma a *n* fluorocromos por ribosoma. Este aumento de la señal de fluorescencia permite mejorar la detección de las células. Al iluminar la muestra a la longitud de onda de excitación del fluorocromo empleado, éste emite a una longitud mayor y se hacen visibles las células marcadas con la sonda. Éstas células sólo corresponderán al grupo filogenético diana, que puede ser desde un dominio - como *Bacteria* - hasta una especie o cepa concreta. Para visualizar y cuantificar el número total de células presentes en la muestra, se usan moléculas fluorescentes como DAPI (4', 6-Diamidino-2-Fenilindol) o SybrGreenI, que se unen inespecíficamente a la doble hélice de DNA.

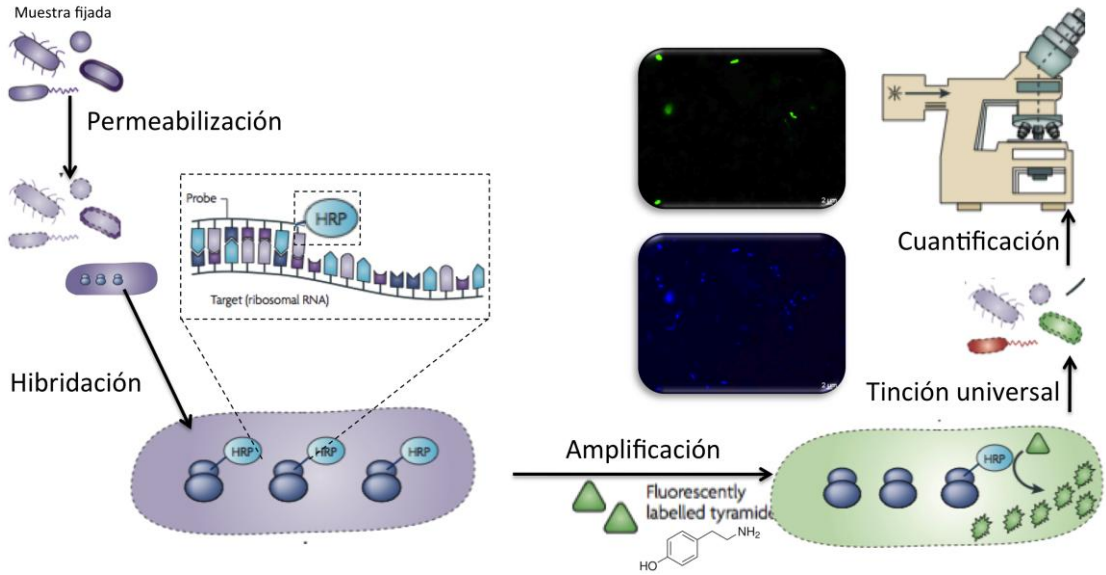

Figura 10: Protocolo de CARD-FISH. La muestra ambiental debe ser fijada *in situ* para preservar el contenido ribosomal. Para permitir el acceso de la sonda con la peroxidasa, las células se permeabilizan. Durante la hibridación, la sonda unida covalentemente a la peroxidasa del rábano (HRP) se une a su región diana del 16S rRNA. En el paso de amplificación, esta enzima cataliza la deposición de las tiramidas unidas a los fluorocromos. Posteriormente, pude teñirse el total de células con colorantes universales y así cuantificar los distintos grupos presentes. Adaptada de (5).

ξ Cultivos de enriquecimiento y aislamiento.

El aislamiento y estudio de cultivos puros supone el complemento ideal del ciclo completo del rRNA. Aunque las técnicas moleculares permiten una rápida caracterización de la diversidad de sistemas complejos, para una completa visión del ecosistema se requieren cultivos de enriquecimiento que prueben la existencia y actividad de los microorganismos que llevan a cabo los metabolismos detectados por técnicas moleculares. Adicionalmente, el aislamiento de los distintos organismos que pueblan un ecosistema es esencial. Los aislados son imprescindibles para estudiar su metabolismo y evaluar tanto su papel en el ecosistema como su potencial biotecnológico.

Los ambientes ácidos están poco caracterizados debido a las peculiaridades fisiológicas de los microorganismos asociados a los mismos (29). En la columna de agua del Tinto, se han estudiado y demostrado metabolismos como la oxidación de compuestos de azufre y hierro, y la reducción de hierro. Muchos de los organismos responsables han sido aislados en distintos ecosistemas ácidos relacionados. Entre ellos, se han aislado gran variedad de quimiolitótrofos oxidadores de hierro y/o azufre como *Acidithiobacillus ferrooxidans*, *Leptospirillum* spp. y *Acidithiobacillus thiooxidans* (36), *Acidithiobacillus caldus* (30), *Sulfobacillus* spp. y miembros de la familia de arqueas *Ferroplasmaceae*. En ambientes ácidos también existe gran variedad de heterótrofos acidófilos como las especies del género *Acidiphilium*, o heterótrofos facultativos como *Acidimicrobium* spp. y *Ferrimicrobium* spp. (34). Estas zonas aerobias de los ambientes ácidos de mina, y los organismos que llevan a cabo la oxidación de los sulfuros metálicos, han sido estudiadas exhaustivamente. Por un lado, su control permitiría reducir el impacto de la contaminación minera, y por otro, poseen gran interés biotecnológico siendo aplicados para biolixiviación de metales.

En cambio, hasta ahora, las zonas anaerobias habían suscitado menos atención y los estudios habían sido limitados principalmente a reducción de hierro (11). Pero en los últimos años se ha

constatado que existen metabolismos, como la reducción de sulfato, capaces de llevarse a cabo a bajos pH y que pueden ser utilizados para la precipitación y recuperación selectiva de los metales pesados, así como bioremediación de zonas contaminadas con radionucleidos (37, 51). También se ha probado la existencia de metanógenas en un ambiente tan hóstil como río Tinto (68). Todo ello, plantea la posibilidad de una mayor diversidad de metabolismos que la inicialmente pensada, y nos muestra la amplitud de nuestro desconocimiento sobre estos ecosistemas haciendo necesario un estudio más exhaustivo de diversas actividades anaerobias.

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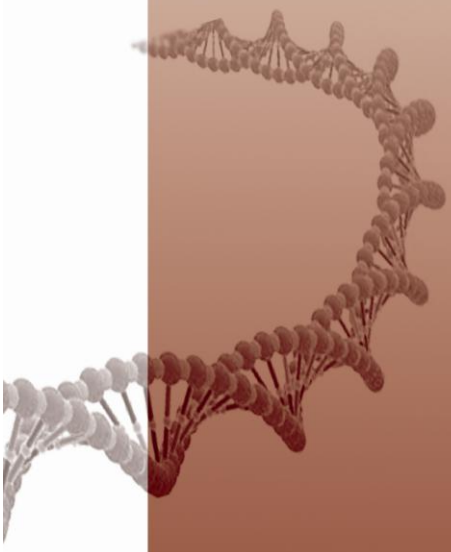
OBJETIVOS

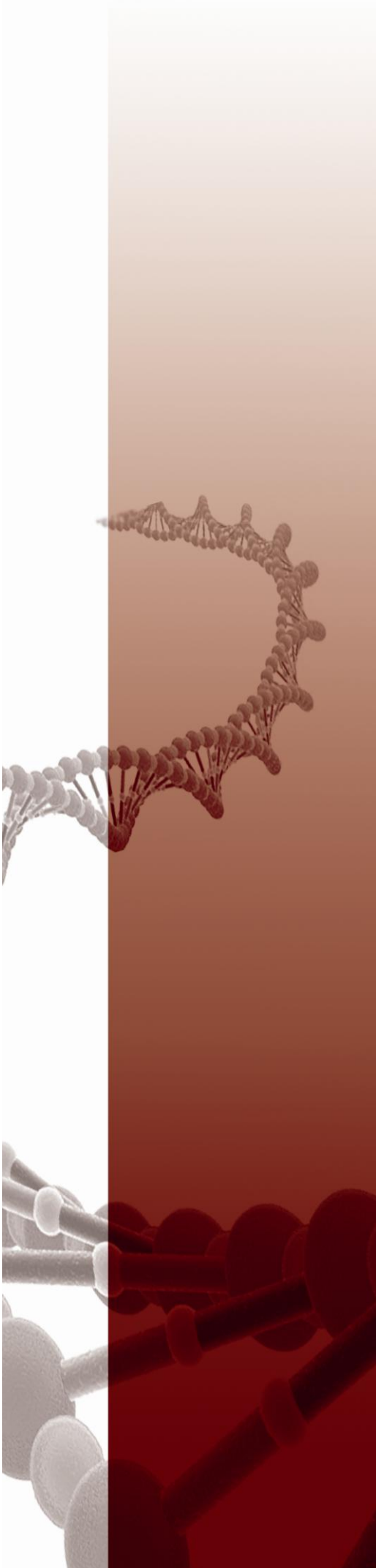


Uno de los grandes objetivos de la microbiología es definir los límites de la vida e identificar los mecanismos que la hacen posible. La exploración de ambientes extremos ha llevado al descubrimiento de numerosos nichos ecológicos que previamente se consideraban inhabitables. Hace, aproximadamente, 20 años comenzó el estudio sistemático de la geomicrobiología del río Tinto mostrando, no solo que la vida era posible en este ambiente extremo, sino que dichas condiciones extremas son fruto de la vida y, por lo tanto, es un ambiente natural y no fruto de la contaminación minera. Con dichos estudios se alcanzó un profundo conocimiento sobre la microbiología y dinámica de los ciclos de los elementos que operan en la columna de agua, pero pocos estudios se habían realizado en las zonas anaerobias del Tinto.

Por tanto, el objetivo principal de la presente tesis es el estudio de la diversidad microbiana de los sedimentos anaerobios de Río Tinto. Para alcanzar este objetivo, se fijaron los siguientes objetivos específicos:

- Adaptación de protocolos estándar de técnicas de ecología molecular (extracción de DNA, CARD-FISH) para el estudio de sedimentos ácidos con alto contenido de metales pesados y bajo contenido celular.
- Diseño, cuando fuera necesario, de sondas específicas para los microorganismos detectados.
- Descripción de la ecología microbiana mediante técnicas de biología molecular como electroforesis en gel de gradiente desnaturalizante (DGGE) y clonaje de genes del 16S RNA ribosómico. A partir de estos datos, inferir las actividades microbianas que podrían tener lugar integrándolas con los datos fisicoquímicos de los sedimentos del río Tinto.
- Identificación y cuantificación de la diversidad microbiana mediante técnicas de hibridación *in situ* (CARD-FISH) en dichos sedimentos para identificar los organismos predominantes y que puedan jugar un papel ecológico relevante.
- Desarrollo de medios de cultivo para anaerobios acidófilos/acidotolerantes.
- Demostración -mediante cultivos de enriquecimiento y medidas de las reacciones metabólicas- de la existencia de las actividades anaerobias inferidas a través de técnicas de biología molecular, e identificación de los organismos responsables.
- Aislamiento de microorganismos como sulfatorreductoras acidófilas con potenciales aplicaciones biotecnológicas.
- Uso de la sulfatorreducción para bioremediación de aguas ácidas de mina.
- Integración de todos los datos obtenidos en un modelo geomicrobiológico que explique el funcionamiento de los ciclos de los elementos en los sedimentos del Tinto.

METODOLOGÍA GENERAL



Los materiales y métodos utilizados han sido descritos y referenciados en cada uno de los capítulos de los que se compone la presente tesis. Sin embargo, debido al formato de las revistas que obligan a una definición breve de los métodos, resulta conveniente una explicación más detallada de aquellos procedimientos que no son rutinarios o han sido puesto a punto exclusivamente en el transcurso de la presente tesis y que por lo tanto, no están debidamente descritos en ninguna publicación previa.

EXTRACCIÓN DE DNA PARA SEDIMENTOS CON METALES PESADOS

Los sedimentos de río Tinto presentan baja densidad celular y un alto contenido en metales pesados que inhiben posteriores procesos de amplificación de DNA. Por ello, para poder obtener una cantidad suficiente de DNA amplificable, fue necesario la aplicación de un tratamiento previo a la extracción (Fig. 1) detallado a continuación.

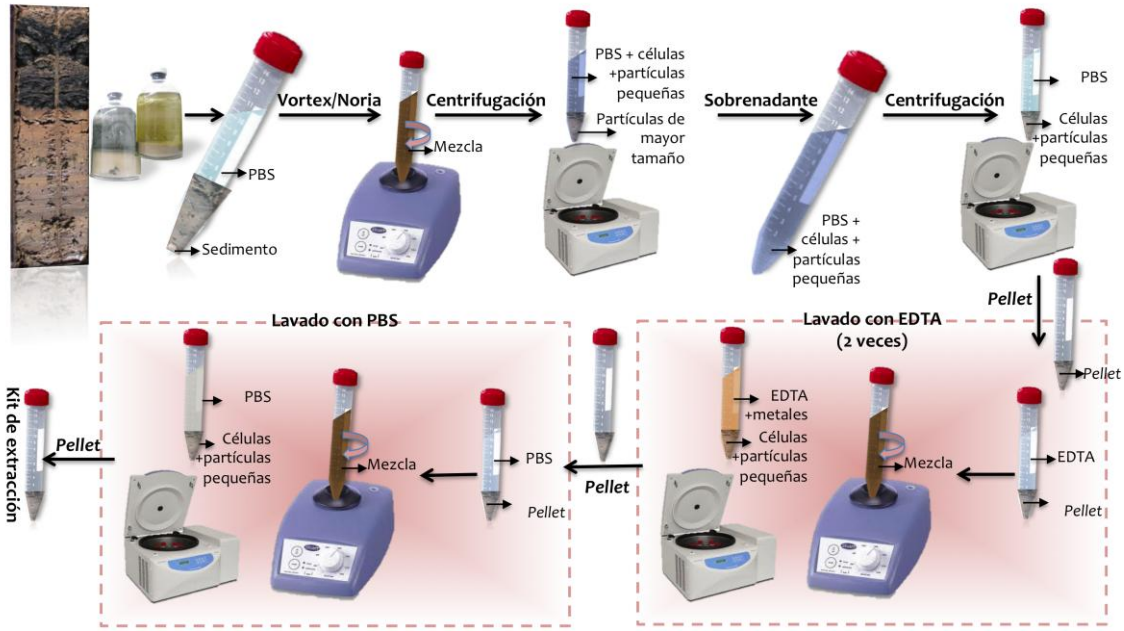


Figura 1: Tratamiento de muestras previo a la extracción de DNA

- Se resuspendió una porción de muestra (~6 gramos de sedimento) en 10 mL de NaCl al 0,9% o PBS (1x) en tubos de 15 mL.
- Se agitó en noria durante 24 horas a 4°C, combinándolo con pulsos de *vortex* (15 min, 3 veces).
- Se centrifugó a 500 x g durante 1 min para retirar las partículas de mayor tamaño.
- Se transfirió cuidadosamente el sobrenadante a otro tubo y se centrifugó de nuevo a 8000 x g durante 20 min.
- Se desechó el sobrenadante.
- Se lavó dos veces el *pellet* con EDTA (0,5 M, pH 8) con objeto de quelar los metales pesados presentes en la muestra.
- Se lavó el pellet con 10 mL de NaCl al 0,9% o PBS (1x) eliminando el exceso de EDTA.
- Se reservó el *pellet* para la extracción de DNA mediante el kit de extracción *FastDNA® SPIN® Kit (For Soil)* (Qbiogene, USA) siguiendo el protocolo indicado por el fabricante a excepción de la adicción de 100 µL de TE (1x) junto con el *PPS* para proteger el DNA de los posibles metales pesados remanentes.

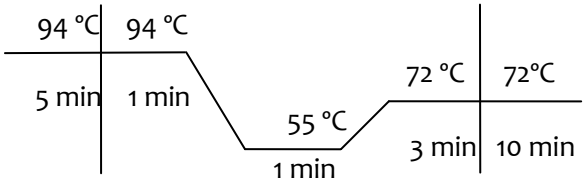
REACCIÓN EN CADENA DE LA POLIMERASA (PCR)

El DNA genómico extraído pudo ser utilizado como molde para reacciones en cadena de la polimerasa (PCRs). La amplificación del gen completo del 16S rRNA se llevó a cabo con los cebadores 27F/1492R para el dominio *Bacteria* y 25F/1492R para *Archaea*, utilizado directamente para clonación o secuenciación de aislados (Tabla 1). Debido a los inhibidores presentes, el DNA era diluido 1:10, añadiéndose 1-3 µL DNA para una PCR de 50 µL. Para DGGE se realizaron PCR anidadas (*nested* PCR) donde el gen completo del 16S RNA fue usado como molde para la amplificación parcial de las regiones variables V3-V5 del gen con los cebadores 341F/907R para *Bacteria* y 622F/1100AR para *Archaea*. Las PCRs se realizaron en un termociclador *Veriti Termal Cycler* (Applied Biosystems, Foster City, CA, EEUU) acorde con los siguientes programas:

16S rDNA, gen completo

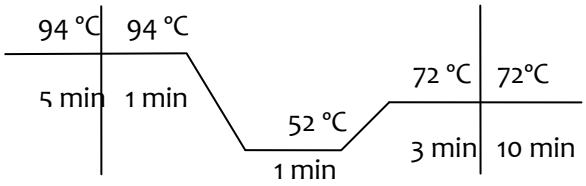
Juego de cebadores: 27F/ 1492R

Diana:…… 16S rDNA *Bacteria*
Mg^{+2}:…… 2,5 mM
$T_{apareamiento}$:…… 55 °C
Programa:



Juego de cebadores: 25F/ 1492R

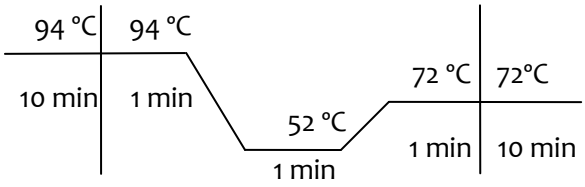
Diana:…… 16S rDNA *Archaea*
Mg^{+2}:…… 3 mM
$T_{apareamiento}$: …… 52 °C
Programa:



16S rDNA, DGGE

Juego de cebadores: 341F /907R

Diana:…… 16S rDNA *Bacteria*
Mg^{+2}:…… 1,5 mM
$T_{apareamiento}$: …… 52 °C
Programa:



Juego de cebadores: 622F/ 1100AR

Diana:…… 16S rDNA *Archaea*
Mg^{+2}:…… 2 mM
$T_{apareamiento}$:…… 44 °C
Programa:

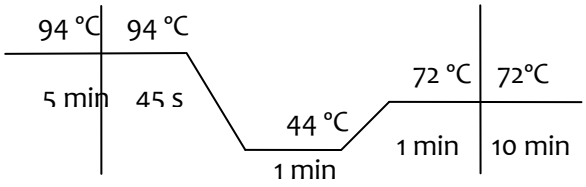


Tabla 1: Secuencias de cebadores utilizados

Cebador [a]	Secuencia (de 5' a 3')
Universal, 16S rRNA	
907RM	CCGTCAATTCMTTTGAGTTT
1492R	TACCTTGTTACGACTT
***Bacteria*, 16S rRNA**	
27F	AGAGTTTGATCMTGGC
341F-(CG)[b]	CCTACGGGAGGCAGCAG
***Archaea*, 16S rRNA**	
25F	CYGGTTGATCCTGCCRG
622F-(GC)[b]	TGAAATCYYRTAATCCC
1100AR	TGGGTCTCGCTCGTTG
Primers	
M13F	GTAAAACGACGGCCAG
M13R	CAGGAAACAGTATGAC

Código IUB (*Internacional Union of Biochemistry*): M = A, C; Y = T, C; R = A, G.
a Las letras F (*forward*) y R (*reverse*) indican la orientación del cebador con respecto al rDNA.
b Para retener el DNA en DGGE, se añade al cebador una secuencia rica en GC en el extremo 5':
5'-CGCCCGCCGCGCCGCGCGGGCGGGGCGGGGGCACGGGGGG-3'

CLONAJE DEL GEN DEL 16S rRNA

ξ Amplificación del gen del 16S rRNA

El gen completo del 16S rDNA fue amplificado según lo descrito previamente. Una alícuota de la PCR fue analizada mediante un gel de agarosa al 1% en TAE (1x) para verificar el correcto tamaño del amplicón. La banda correspondiente al gen del 16S rDNA (~1500 pb) se recortó minimizando la exposición al ultravioleta y se purificó con GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) para eliminar dímeros de *primers* u otros productos inespecíficos.

ξ Ligación

El producto purificado de PCR fue ligado a un vector pGEM-T vector (Promega, Madison, WI) en las siguientes condiciones: 5 µL *rapid ligation buffer* (2x) + 1 µL pGEM-T vector + 3 µL producto de PCR + 1 µL T4 DNA ligase. Los 10 µL de la reacción se mezclaron cuidadosamente e incubaron a 4 ºC durante la noche (para aumentar la eficiencia de ligación) en un tubo de 0,5 mL (con menor adhesividad de DNA). Es crucial agitar en un *vortex* vigorosamente el tampón suministrado por la casa comercial antes de ser utilizado y alicuotarlo individualmente para evitar que posteriores congelaciones/descongelaciones degraden el ATP presente.

ξ Transformación

Los distintos fragmentos de DNA conteniendo el gen para el 16S rRNA fueron separados por transformación de células competentes *Escherichia coli* DH5α siguiendo el siguiente protocolo:

- Se añadieron 5 µL de la mezcla de ligación.
- Se incubaron 30 min en hielo.
- Se les sometió a un choque térmico durante 1,5 min a 42ºC.
- Se incubaron 2 min en hielo.
- Se añadieron 500 µL de medio SOC.
- Se incubaron durante 2 horas en agitación fuerte a 37ºC.

- Se sembraron distintas cantidades (25, 50 y 75 µL) en las placas de LB/Amp/X-gal.
- Tras una noche de crecimiento a 37ºC, los clones recombinantes fueron identificados como colonias blancas y transferidos a nuevas placas para asegurar la correcta asignación positiva de los clones.

ξ **Cultivo**

Los clones que resultaron positivos fueron crecidos en 1,5 mL de medio *Terrific Broth* (Sigma-Aldrich) en agitación a 37ºC de 12-16 h en placas de 96 pocillos.

ξ **Lisis alcalina**

El DNA de los plásmidos recombinantes fue extraído mediante lisis alcalina (1).

- Se centrifugaron las placas a 3750 rpm durante 20 min. Se volcaron para eliminar sobrenadante.
- Se añadió al pellet 300 µL de TE (1x) y se resuspendió con *vortex*.
- Se centrifugó (3750 rpm) durante 20 min. Se eliminó el sobrenadante.
- Se añadió al *pellet* 100 µL de la solución 1 (*fría*) y se resuspendió con *vortex*.

  SOLUCIÓN 1: 50 mM glucosa; 25 mM EDTA (pH 8, 0,5 M); 25 mM Tris-Cl (pH 8, 1 M). Para preparar 100 mL se deben mezclar 4 mL EDTA (pH 8, 0,5 M)+ 1 g glucosa + 2,5 mL Tris-Cl (pH 8, 1 M)+ 93 mL agua milli-Q y esterilizar a 0,5 atm durante 30 min.
- Se añadió al *pellet* 200 µL de la solución 2. Se tapó la placa e invertió 2-3 veces sin agitar.

  SOLUCIÓN 2: 0,2 N NaOH; 1% SDS. Es necesario preparar en el momento mezclando 800 µL de un *stock* NaOH (5 N) con 2000 µL de SDS (10%) y 400 µL de agua milli-Q. Esterilizar por filtración con filtros de 0,22 µm (MilliPore).
- Se incubó en hielo a 4ºC durante 5 min.
- Se añadió 150 µL de la solución 3. Se tapó e invirtió 2-3 veces hasta que se enturbió.

  SOLUCIÓN 3: Mezclar 60 mL de NaAc (5 M) con 11,5 mL HAc glacial y 28,5 mL agua milli-Q. Esterilizar por filtración con filtros de 0,22 µm (MilliPore).
- Se incubó en hielo 10 min.
- Se centrifugó a 3750 rpm durante 45 min a 4ºC.
- Se transfirió el sobrenadante a una placa nueva.
- Se precipitó el DNA con 1 mL de isopropanol (previamente a –20ºC) *overnight* a -20ºC.
- Se centrifugó a 3750 rpm durante 45 min a 4 ºC.
- Se eliminó el sobrenadante suavemente con pipeta y se añadieron, sin resuspender, 150 µL de etanol al 70% (previamente enfriado a –20ºC).
- Se centrifugó a 3750 rpm durante 30 min a 4 ºC.
- Se eliminó el sobrenadante cuidadosamente.
- Se secó completamente el *pellet* al aire durante 1 h o en un *speed-vac* durante 10 min.
- Se resuspendió el *pellet* en 30 µL de agua milli-Q con RNAsa (50 µg mL^{-1}). Por placa, se añade 30 µL de RNAsa (10 mg mL^{-1}) a 3000 µL de agua milli-Q.
- Se incubó 30 min a 37ºC.
- Se conservó a -20 ºC.

El tamaño correcto de los insertos fue verificado mediante electroforesis convencional en geles de agarosa a 0,8 % p/v en TAE (1x) cargando 3 µL del DNA plasmídico (Fig. 2). Los

falsos positivos se distinguieron por su menor peso molecular al no tener inserto el 16S rRNA (1500 pb).

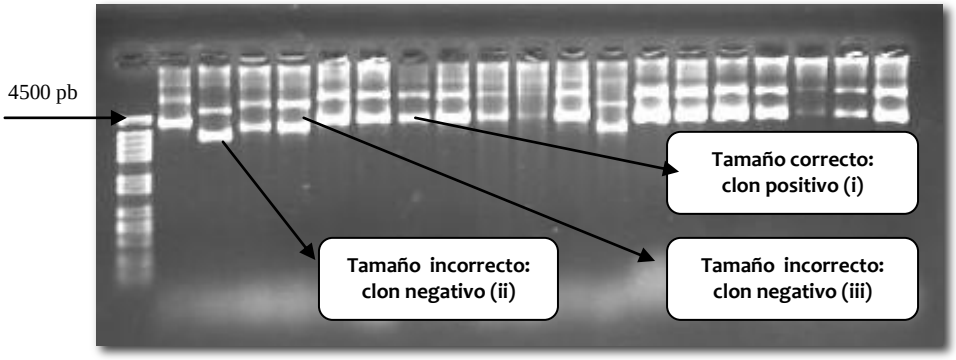


Figura 2: Comprobación del tamaño de los insertos en los plásmidos. El plásmido utilizado contiene 3015 pares de bases (según la información facilitada por el fabricante) que, unidos a los 1465 pb del inserto suman casi 4500 pb. Si comprobamos la extracción del DNA plasmídico mediante electroforesis convencional, el plásmido unido al inserto da como resultado una banda del tamaño de (i). Aquellos plásmidos en los que no se ha insertado nada generan una banda tipo (ii), y en los que se ha insertado algo, pero de menor tamaño, como por ejemplo dímeros de cebadores, la banda resultante es del tipo (iii).

ξ Digestión del DNA plasmídico

Si el número de clones a analizar es muy alto, el coste de secuenciación puede ser elevado, de modo que, mediante agrupamiento por digestión, se redujo el número de clones a secuenciar. Para ello, el DNA plasmídico de los clones positivos se digirió enzimáticamente (*Amplified Ribosomal DNA Restriction Analysis*, ARDRA) usando la endonucleasa BfuCI (1 U, 4 h, 37 °C) mezclando 10 µL agua milli-Q + 1,5 µL tampón Bfu (10x) + 0,36 µL enzima BfuCI + 0,15 µL BSA (100x) + 3 µL DNA plasmídico. Los fragmentos resultantes fueron analizados en un gel de agarosa al 3% p/v mediante electroforesis convencional a 40 mA durante 2 h (Fig. 3). Los clones fueron agrupados según los distintos patrones de restricción obtenidos. Se secuenciaron dos representantes de cada grupo utilizando las pareja de cebadores M13F/M13R, secuencias plasmidicas que flanquean la zona de inserción del gen del 16S rRNA. En caso necesario se utilizaron cebadores internos cuya diana se encuentra en el 16S rDNA (341F para *Bacteria*, 622F para *Archaea*).

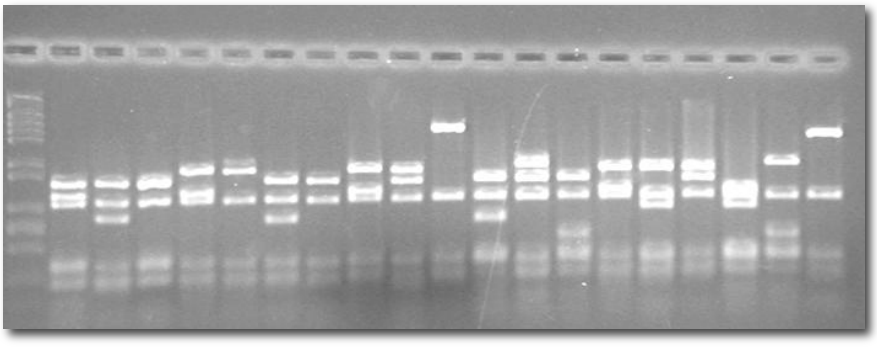


Figura 3: Patrones de restricción obtenidos con la enzima Bfu CI.

HIBRIDACIÓN IN SITU CON SONDAS GENÉTICAS (CARD-FISH)

Se utilizó una variante de la hibridación *in situ* con sondas fluorescentes (FISH), llamada CARD-FISH, que emplea un paso de amplificación enzimática de la señal fluorescente. El protocolo utilizado está basado en el descrito por Pernthaler y col. 2002 (2) con modificaciones para adaptarlo a los complejos sedimentos del río Tinto. Como notas generales cabe destacar que la autofluorescencia emitida por las partículas de sedimento no permitió estudiar estas muestras bajo las condiciones de FISH convencional. Además se requirieron protocolos especiales para la tinción inespecífica: el colorante universal DAPI no pudo ser utilizado eficientemente para el conteo total del número de células, teniendo que utilizar como alternativa Sybr Green I.

La tiramida-Alexa 488 y SybrGreen I emiten en la misma longitud de onda de modo que para relativizar los conteos de sondas respecto a un numero absoluto, hubo que hacerlo respecto al número total de células hibridadas con la sonda específica para bacterias en vez de contra el número total de células. Otras combinaciones de tiramidas y tintes no fueron posibles al no emitir suficiente señal para distinguirse del fondo.

Cabe también señalar que, en algunos casos, el número de células era demasiado bajo para permitir el conteo específico. Por ello, se llevó a cabo una centrifugación en gradiente de densidad que permitiera separar las células del sedimento y, tras filtrar la banda donde se sitúan las células, disponer de mayor número de células por filtro.

ξ **Fijación**

Con el fin de conservar el contenido ribosomal de la comunidad microbiana, las muestras se fijaron inmediatamente tras sacar el testigo de sedimento. El procedimiento de fijación incluyó la adicción de formaldehído a la muestra a una concentración final del 4 % en 0,9 % NaCl. Se incubó en hielo durante 4 horas para después lavar las muestra dos veces con PBS (1x) y resuspender finalmente el PBS:Etanol (1:1) diluyendo aproximadamente 1 gramo de sedimento por 1,5 mL de PBS:Etanol.

ξ **Sonicación**

- Se diluyeron las muestras 1:10 en 1 mL PBS:Etanol (1:1) en tubos *eppendorf* de 2 mL y se mezclaron mediante *vortex*.
- Se dispusieron las muestras sobre hielo y se sonicaron con una sonda Sonic (Bandelin, Sonopuls HD200) 3 veces, 20 s a 20% de intensidad cada sonicación.

ξ **Centrifugación en gradiente de densidad**

- Se dispusieron las alicuotas sonicadas en tubos *eppendorf* de 2 mL.
- Se incorporó debajo, muy cuidadosamente para evitar mezcla, 1 mL de solución de Hystodenz (60% p/v en PBS, densidad 1,3) (Sigma-Aldrich) usando una jeringa larga.
- Se centrifugó la muestra a 14000 x g durante 90 min a 4°C.
- Se tomó la fracción superior del Hystodent con la fracción celular de la muestra y se filtró como el resto de muestras.

ξ **Preparación de los filtros**

- Se filtraron de 40 a 100 µL de la muestra (diluyendo en 10 mL de PBS) a través de filtros de policarbonato (GTTP, 0,2 µm de tamaño poro, Millipore) con el lado brillante hacia arriba a un vacío de 0,5 – 1 mbar. Bajo este filtro, se coloca un filtro humedecido

en PBS de Celulosa-Nitrato (HAMP, 0,45 µm de tamaño poro, Millipore) para homogeneizar la presión y propiciar la homogeneidad de la muestra-

- Se dejaron secar los filtros al aire en placas Petri. Es importante teñir la muestra y comprobar que la dilución sea la adecuada, como regla general debería haber unas 50-100 células por campo o deberían estar cubiertos unos 2/3 del filtro con sedimento.

ξ **Tincion inespecífica**

- DAPI: las secciones se colocaron boca-arriba sobre los porta-objetos y se embebieron en 8 µL de la mezcla de DAPI/Citifluor (1 µg mL^{-1}). Se dejaron al menos 30 min a -20°C para intensificar la señal del DAPI.
- SybrGreen I: Las secciones se colocaron boca-arriba sobre los porta-objetos y se embebieron en 8 µL de la mezcla de SybrGreen/Moviol Medium (1:40) preparado según lo descrito en Lunau and col., 2005 (3). Se dejaron al menos 30 min a -20°C para intensificar la señal.

ξ **Inclusión de los filtros en agarosa**

- Se precalentó agarosa LMP (*low melting point*) 0,1 % en microondas hasta ebullición, y se atemperó hasta ~50°C.
- Se dispuso en un spray y se roció el filtro 2-3 veces a 50 cm de distancia.
- Se dejaron secar los filtros con las placas abiertas durante 20-30 min a 37°C hasta que la agarosa estuvo seca.

ξ **Inactivación de las peroxidasas endógenas**

- Se incubaron los filtros boca-arriba en H_2O_2/metanol [0,15% H_2O_2/metanol = 50 µL H_2O_2 (30%) en 10 mL metanol] durante 30 min a temperatura ambiente en placa Petri sellada con *parafilm* o en campana de extracción para evitar el riesgo de inhalación por el extenso periodo de exposición.
- Se lavaron los filtros en agua durante 1 min a temperatura ambiente.
- Se lavaron los filtros en etanol absoluto durante 1 min a temperatura ambiente.
- Se dejaron secar completamente al aire.
- Se cortaron con bisturí y se rotularon con lápiz blando (HB, 2B).

ξ **Permeabilización**

con lisozima (recomendada para mayoría de las bacterias)

- Se preparó una solución fresca de lisozima [10 mg mL^{-1} en EDTA (0,05 M, pH 8); Tris-HCl (0,1 M, pH 8) = 8 mL agua milli-Q + 1 mL Tris-HCl (0,1 M, pH 8) + 1 mL EDTA (0,05 M, pH 8) + 100 mg lisozima].
- Se incubaron los filtros boca-abajo durante 1 h.
- Se lavaron los filtros con agua milli-Q durante 1 min a temperatura ambiente.

con acromopeptidasa (recomendada para Firmicutes)

- Se incubaron los filtros durante 30 min a 37°C boca-abajo en acromopeptidasa (60 U mL^{-1}) = 9860 µL agua milli-Q + 20 µL NaCl (5 M) + 100 µL Tris-HCl (1 M, pH 8) + 20 µL stock acromopeptidasa (30000 U mL^{-1}).

con Proteinasa-K (recomendada para arqueas):

- Se preparó una solución de trabajo de Proteinasa-K [15 µg mL^{-1} en EDTA (0,05 M, pH 8); Tris-HCl (0,1 M, pH 8) = 8 mL agua milli-Q + 1 mL Tris-HCl (0,1 M, pH 8) + 1 mL EDTA (0,05 M, pH 8)+ 7,5 µL stock Proteinasa (20 mg mL^{-1})].
- Se incubaron los filtros durante 3 min a 37 °C.
- Se inactivó la Proteinasa-K en 10 mL de HCl (0,1 M) durante 1 min a temperatura ambiente.
- Se lavaron los filtros en agua milli-Q y se dejaron secar completamente al aire.

ξ **Hibridación**

- Se mezcló el tampón de hibridación (Tabla 2) con la solución de trabajo de la sonda (50 ng μL^{-1}) en un ratio de 300:1 v/v en un tubo eppendorf de 2 mL.
  - **Tabla 2: Tampón de Hibridación para sondas marcadas con HRP**

Reactivos	Volumen 10 ml
5 M NaCl	1800 µL
1 M Tris-HCl pH 8	200 µL
Sulfato de Dextrano	1 g
Formamida (x%)	0 µl - 0% /6000 µL - 60%
Agua milli-Q	6990 µL - 0% / 990 µL - 60%
Agente bloqueante*	1000 µL
SDS (10%)	10 µL

  - *Agente bloqueante: 10% en tampón de ácido maleico.
  - Tampón de ácido maleico: 100 mM de ácido maleico; 150 mM NaCl (pH 5)
- Se dispusieron las secciones de filtro pegadas a los laterales del *eppendorf* con la cara con muestra hacia el interior para evitar desprendimiento de la misma.
- Se hibridaron las muestras estáticamente a 46°C durante 2 h.

ξ **Lavado**

- Se preparó y precalentó el tampón de lavado a 48°C.
- Se lavaron los filtros durante 10 min a 48°C.
- Se incubaron las secciones en 50 mM de PBS (1x) durante 15 min a temperatura ambiente para equilibrar la sonda con la peroxidasa.

ξ **Amplificación**

- Se preparó una solución stock fresca de H_2O_2 (100x) [0,15 % H_2O_2/PBS (1x) = 1 µL H_2O_2 30% en 200 µL PBS (1x)].
- Se mezclaron 1 mL del tampón de amplificación (Tabla 3) con 10 µL de la solución anterior de H_2O_2 (100x) y 1 µL de la tiramida marcada con el cromóforo fluorescente Alexa 488 (1 mg fluorocromo mL^{-1}) en un tubo de 2 mL donde se introdujeron cuidadosamente las secciones de filtro.
- Se incubaron durante 45 min a 46°C en oscuridad.
- Se transfirieron sucesivamente las secciones a 50 mL de agua milli-Q y a etanol 96%.
- Se secaron las secciones al aire.
- Se tiñeron inespecíficamente con DAPI según lo descrito anteriormente.
  - **Tabla 3: Tampón de Hibridación para sondas marcadas con HRP**

Reactivos	Volumen 50 ml
5 M NaCl	12 mL
PBS (10x)	4 mL
Agente bloqueante 10%*	0,4 mL
Sulfato de Dextrano	4 g

ξ Análisis y cuantificación de células por microscopía

Las muestras hibridadas fueron observadas con un microscopio de epifluorescencia y cuantificadas manualmente. Los cálculos porcentuales se realizaron frente al número de células hibridadas con la sonda específica de bacteria en vez de contra el número de células totales. Se contaron alrededor de 1000 células hibridadas por muestra cuando era posible, sino era el caso, al menos 200 campos.

Para estimar número total de células por gramo de sedimento presentes en la muestra original a partir del número de células contadas por campo se consideraron los siguientes parámetros y sus valores:

Área del filtro: $1{,}93 \cdot 10^{8}$ µm^2.

Área de la rejilla contada (microscopio): $1{,}5 \cdot 10^{4}$ µm^2.

Correspondiendo el peso y volumen de muestra a la relación 1 g: 1,5 mL PBS:etanol.

Y se aplicó la siguiente fórmula.

$$N = media\,células * factor\,dilución * \frac{área\,filtro}{área\,rejilla} * \frac{volumen\,muestra}{volumen\,filtrado} / peso\,muestra$$

ξ Diseño de sondas

Los altos porcentajes de hibridación obtenidos para la sonda específica de *Deltaproteobacteria*, la abundancia de clones pertenecientes a *Desulfurella* obtenidos durante este trabajo unido a la ausencia de una sonda específica de *Desulfurella* previamente diseñada, hizo necesario el diseño de una nueva sonda para este género. Para diseñar una nueva sonda es preciso seleccionar una secuencia de oligonucleótidos completamente específica, es decir, complementaria a una región de las secuencias diana y que se diferencie en, al menos, un nucleótido (*mismatch*) de la misma región del resto de secuencias (organismos) no diana. La sonda específica para el género *Desulfurella* fue diseñada usando la función PROBE_DESIGN del paquete informático ARB. Para ello,

- Se seleccionaron como secuencias diana aquellas secuencias de *Desulfurella* obtenidas en los clonajes y DGGE y todas las secuencias de la base de datos de ARB incluidas en el género.
- Se comprobó y mejoró el alineamiento de las secuencias diana manualmente en la ventana ALIGN de ARB.
- Con las secuencias diana marcadas, se abrió la ventana PROBE DESIGN.
- Se seleccionó el PT_SERVER actualizado y se definieron los parámetros: "minimun percentage of group hits" a 100, "máximum number of nongroup hits" a 0 y la longitud de la secuencia se dejó, por defecto, en 18 nucleótidos.
- Se presionó el botón GO. Los resultados aparecen en la ventana PD RESULT (Fig. 4), mostrando las potenciales sondas y sus parámetros asociados como el largo de la secuencia, la temperatura de disociación, el contenido G+C y la localización respecto a *Escherichia coli*.

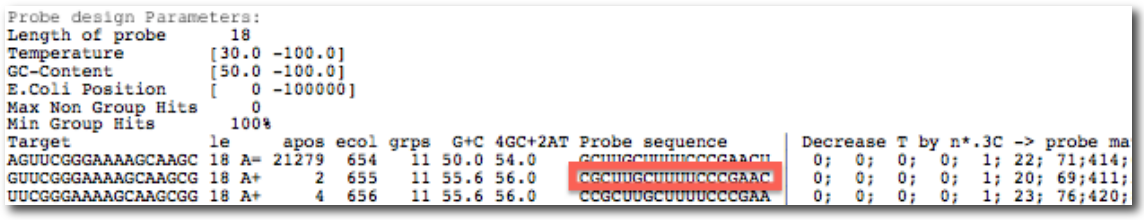


Figura 4: Ventana PD RESULT del paquete informático ARB.

Su especificidad fue evaluada con la función PROBE_MATCH comprobando que poseía de 2 a 3 *mismatches* con el resto de grupos. Tras el marcaje de las sondas con HRP, las condiciones de hibridación fueron optimizadas variando la concentración de formamida de 0 a 60 % en intervalos de 5 % siguiendo el protocolo descrito para CARD-FISH. La especificidad de las sondas fue evaluada con controles positivos y negativos. Se utilizaron como controles positivos la cepa tipo del género: *Desulfurella kamchatkensis* (DSM 10409). Como controles negativo se utilizaron cultivos de *Syntrophobacter wolinii* (DSM 2805) and *Acidobacterium capsulatum* (DSM 11244). Se seleccionó el máximo porcentaje en el cual la señal de formamida era óptima pero sin señal para los controles negativos (35%).

PREPARACIÓN DE MEDIOS ANAEROBIOS

El cultivo de organismos anaerobios presenta ciertas dificultades derivadas de la toxicidad que presenta el oxígeno para dichos organismos. Durante la presente tesis se han desarrollado diversos medios, explicados en los capítulos 3, 4 y 5 pero una explicación detallada del protocolo es necesaria para la correcta reproducibilidad. Se explicará en detalle el medio para sulfatorreductoras acidófilas por ser el más complejo y con mayores aplicaciones.

ξ **Composición de las soluciones stock (por litro)**

Solución 1: 27,2 g KH_2PO_4

Solución 2: 35,6 g $Na_2HPO_4 \cdot 2H_2O$

Solución 3A: 24 g NH_4Cl; 24 g NaCl; 8 g $MgCl_2 \cdot 6H_2O$

Solución 3B: 11 g $CaCl_2 \cdot 2H_2O$

Trace elements:

Solución Stock Ácida (I)

50 mM HCl	18 g
1 mM H_3BO_3	61,8 mg
0,5 mM $MnCl_2$	61,25 mg
7,5 mM $FeCl_2$	943,5 mg
0,5 mM $CoCl_2$	64,5 mg
0,1 mM $NiCl_2$	12,86 mg
0,5 mM $ZnCl_2$	67,7 mg

Solución Stock Básica (II)

10 mM NaOH	400 mg
0,1 mM Na_2SeO_3	17,3 mg
0,1 mM Na_2WO_4	29,4 mg
0,1 mM Na_2MoO_4	20,5 mg

Vitaminas: 20 mg Biotina; 200 mg nicotinamida ; 100 mg ácido p-aminobenzoico; 200 mg tiamina (vitamina B1); 100 mg ácido pantotenoico; 500 mg piridoxamina; 100 mg cianocobalamina (vitamina B12); 100 mg riboflavina.

Solución 7: 240,2 g $Na_2S \cdot 9\ H_2O$ (almacenado en oscuridad y anaerobiamente)

Solución 8: 0,5 g resazurina

Sal de Mohr (200x): 10 g of $(NH_4)_2\ FeSO_4$ en 100 mL. Filtrar.

ξ **Preparación del medio (para un litro).**

- Se añadieron las siguientes cantidades en un matraz *Erlenmeyer*:

  500 mL agua destilada
  1 mL solución 8
  15 mL solución 1
  15 mL solución 2
  12,5 mL solución 3A
  1 mL solución traza I
  1 mL solución traza II
  1,42 g Na_2SO_4
  0,1 g extracto de levadura

- Se llevó hasta 950 mL con agua destilada.
- Se llevó hasta ebullición para eliminar el oxígeno disuelto (no más de 20 segundos).
- Se enfrió en hielo gaseando el medio con N_2.
- Se ajustó el pH del medio según la tabla 4:

Tabla 4: Relación de pH

pH antes de autoclavar	pH deseado tras autoclave
3,85	4
4,75	4,5
5,55	5
6,4	5,5
6,7	6

- Se alicuotó en viales o botellas de suero (dejando el 50% de la botella libre).
- Se cerraron las botellas con tapón y septum.
- Se intercambió la fase gaseosa con N_2/CO_2 o H_2/CO_2 a una presión de 1,5 atm.
- Se autoclavaron las botellas durante 20 min a 121°C (1 atm de sobrepresión).
- Se autoclavaron 10 mL de sol 3B.
- Se autoclavaron 50 mL de agua milli-Q.

Tras autoclavar:

- Se añadió 1 mL de la solución de vitaminas esterilizando por filtración a los 10 mL de sol 3B y se añadió 0,5 mL de esta solución a cada botella con 45 mL de medio.
- Se añadió 1 mL de la sol 7 esterilizada por filtración a 50 mL del agua milli-Q esteril para hacer una solución reductora y se añadieron 2,5 mL de esta solución a cada botella con 45 mL de medio.
- Se añadieron las fuentes de carbono: 0,250 mL de las soluciones stock (1 M) estériles hasta una concentración final de 5 mM.

ξ **Placas con agar:**

- Se siguió el protocolo anterior sustituyendo la solución 7 (reductora) por L-cisteína (0,5 g L^{-1}) y añadiendo agar al 0,8 % (Noble Agar, Difco).

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3. **Lunau, M., A. Lemke, K. Walther, W. Martens-Habbena, and M. Simon**. 2005. An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. Environ. Microbiol. **7**:961-968

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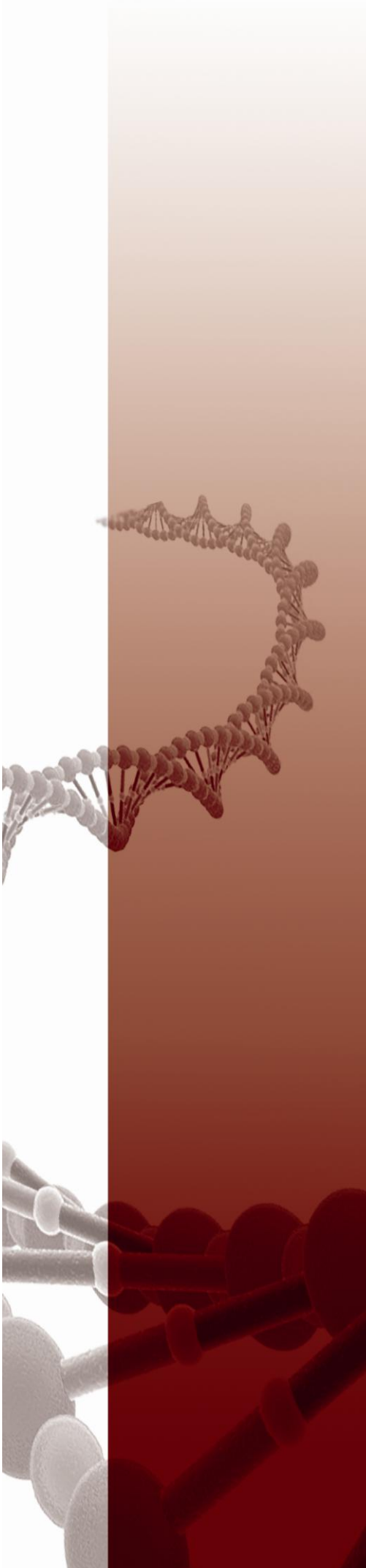


Los capítulos que integran este proyecto de tesis doctoral fueron redactados íntegramente en inglés acorde con los requisitos de publicación en revistas de ámbito internacional. A continuación se enumeran los artículos producidos en el transcurso de la presente tesis: título, autores y estado de publicación.

Sánchez-Andrea I., N. Rodriguez, R. Amils, and J. L. Sanz.
Microbial Diversity in Anaerobic Sediments at Rio Tinto, a Naturally Acidic Environment with a High Heavy Metal Content.
Applied and Environmental Microbiology (2011) **77:**6085-6093.

Sánchez-Andrea I., K. Knittel, R. Amann, R. Amils, and J. L. Sanz.
Quantification of Tinto River Sediments Microbial Communities: the Importance of Sulfate-Reducing Bacteria and their Role in Attenuating Acid Mine Drainage. *Applied and Environmental Microbiology* (2012) **78 (13):** doi: 10.1128/AEM.00848-12

Sánchez-Andrea I., P. Rojas, R. Amils, and J. L. Sanz.
Screening of Anaerobic Activities in Sediments of an Acidic Environment: Tinto River
Aceptado en *Extremophiles* el 25-Abril-2012: EXT-12-Feb-0043.R1

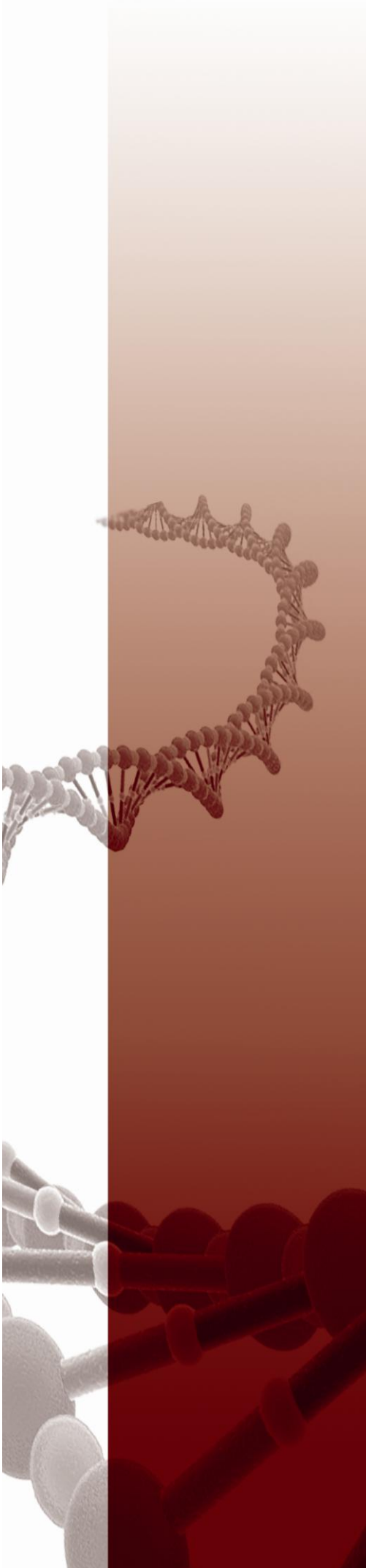
Sánchez-Andrea I., A.J.M. Stams, R. Amils and J.L. Sanz.
Enrichment and isolation of acidophilic sulfate-reducing bacteria from Tinto River sediments
Environmental Microbiology reports (Subscrito Junio 2012. En revisión)

Sánchez-Andrea I., D.Triana and J.L. Sanz
Bioremediation of acid mine drainage coupled with domestic wastewater treatment.
Water Science and Technology (Subscrito Abril 2012. En revisión)

Sánchez-Andrea I., N.Rodriguez, R. Amils, and J. L. Sanz
Microbial diversity of anaerobic zones of Tinto river combining culture dependent and non-dependent methods.
En preparación.

Amils, R., E. Gonzalez-Toril, A. Aguilera, N. Rodriguez, D. Fernandez-Remolar, F. Gomez, A. Garcia-Moyano, M. Malki, M. Oggerin, I. Sánchez-Andrea, and J. L. Sanz.
From Río Tinto to Mars: the terrestrial and extraterrestrial ecology of acidophiles.
Advances in Applied Microbiology (2011) **77:**41-70.

CHAPTER 1: Microbial Diversity of Anaerobic Sediments of Río Tinto: a Natural Acid and High Heavy Metals Content Environment



INTRODUCTION

Pyrite (FeS_2) is the most abundant sulfide mineral in Earth's crust. When it is exposed to air and water, its oxidation can be carried out with either atmospheric oxygen or ferric iron, according to the equations 1 to 3.

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad \text{(eq. 1)}$$

$$Fe^{2+} + H^+ + 0.25O_2 \rightarrow Fe^{3+} + 0.5H_2O \quad \text{(eq. 2)}$$

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

Ferric iron has been shown to oxidize pyrite 18 to 170 times more rapidly than O_2, producing waters with a low pH and a high iron content. However, this step is limited by the rate of ferrous iron oxidation (equation 2), which is greatly increased (by up to 5 orders of magnitude) at low pH through the action of Fe-oxidizing chemolithoautotrophs (38), such as the bacteria *Acidithiobacillus* spp. and *Leptospirillum* spp. Furthermore, low pH facilitates metal solubilization, particularly that of cationic metals; therefore, acidic water tends to be highly metalliferous (24). Pyrite and ferrous iron oxidation are both processes that occur naturally and, indeed, natural acidic drainage has been identified in many locations (44). Mining in these areas is known to dramatically increase the ferrous iron oxidation rates (39).

The Tinto River is a natural acidic drainage environment. It rises in Peña de Hierro, in the core of the Iberian Pyritic Belt (IPB), and reaches the Atlantic Ocean at Huelva, Spain. The IPB is one of the largest massive sulfidic deposits on Earth (250 km-long) and formed as a hydrothermal deposit during the Paleozoic accretion of the Iberian Peninsula (32). Although the IPB has been subjected to mining activities for thousands of years (45), a recent analysis has shown that the iron oxides present in its sedimentary deposits are the result of the metabolic activity of acidophilic prokaryotes (14). The Tinto River is an unusual extreme ecosystem due to its size (100 km long), constant acidic pH (mean pH, 2.3) and high concentration of heavy metals, iron and sulfate in its waters, characteristics that make Tinto River Basin comparable to Acidic Mine Drainage (AMD) systems.

The Tinto River has been the focus of an increasing amount of research including biogeochemistry (exploring the nature of the microbial communities associated with AMD), biotechnology (bioleaching processes), microbiology (acidic environments offer an almost unique opportunity to reveal habitat biological complexity) and Mars-analogue studies (jarosite is a common mineral on both Mars and Earth). Consequently, much is already known about the microbiota inhabiting the water column (1, 15, 18, 43, 52). Microbial ecology studies have shown that ca. 80% of the prokaryotic diversity in the water is explained by the presence of three bacteria *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans* and *Acidiphilium* spp., all of which are all involved in the iron cycle. Some other species related to the iron cycle have also been identified (i.e. *Ferroplasma acidiphilum* or *Ferrimicrobium acidiphilum*), but their low number suggests that they play a minor role in this cycle (18). Despite the low prokaryotic diversity, an unexpected degree of eukaryotic diversity has been found, and is the principal contributor of biomass in this hostile river (1, 52).

Despite ecological interest in the underlying sediments, they have been studied only very sparsely and studies have mainly focused on methanogens (46). So far, no complete studies of the anaerobic microbial diversity were undertaken. In this paper we present an extensive survey of the Tinto River anaerobic sediment microbiota, using culture-independent

approaches targeting the small-subunit (SSU) rRNA such as PCR-denaturing gradient gel electrophoresis (DGGE) and cloning of 16S rRNA genes. Furthermore, a model of the iron, sulfur and carbon cycles is proposed to elucidate the microbial ecology of AMD-related sediments.

MATERIALS AND METHODS

Field site description and sampling

The acidic sediments studied are located in Tinto River basin (Huelva), in southwestern Spain. Samples were collected from two sampling sites, *SN* dam (Universal Transverse Mercator [UTM], E715378-N4178002) and *JL* dam (UTM: E715073-N4174390), in March 2008 and March 2009. Sediment cores (inner diameter, 7 cm; length, 45 cm) were taken with a sampler (Eijkelkamp Agrisearch equipment, Giesbeek, The Netherlands). The redox potential (E) and pH of the drill core samples were measured *in situ* with E and pH probes connected to a Thermo Orion 290A potentiometer placed into the fresh sediment just after extracting the core. The cores were sliced according to both physicochemical gradients and visual aspects, and the slices were kept separately until processing in the laboratory.

Nucleic acid extraction from sediments

Total DNA was extracted from 6 g of each slice of sediment using FastDNA® SPIN® Kit (for Soil) (Qbiogene, USA). Adaptation of the commercial protocol was carried out to optimise it for sediments with high concentrations of heavy metals and low biomass content. Samples were first resuspended in 10 mL of phosphate-buffered saline (PBS) and sonicated to detach cells from the solid phase. The sediment was centrifuged at 500 x g for 1 min in order to remove larger particles; the supernatant was carefully removed and centrifuged again at 8000 x g for 20 min. After the supernatant was discarded, the remaining pellet was resuspended in 10 mL 0.5 M EDTA, pH 8 and incubated overnight at 4°C to dissolve humic substances and remove heavy metals. After incubation, sediment was washed with PBS and DNA extraction procedures were applied to the pellet.

Denaturing gradient gel electrophoresis (DGGE) from sediment samples

DGGE analyses were carried out in samples from March 2008 to get a first insight of the microbial diversity of the sediments. The V3 to V5 variable regions of the 16S rRNA gene were amplified with the primers set 341F (GC)-907R (annealing temperature (Ta=52°C) for *Bacteria* and 622F (GC)-1100R (Ta =42°C) for *Archaea*. Primers 341F (GC) and 622F (GC) included a GC clamp: 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCC CCG CCC-3' (37). The amplification reaction was performed according to the *Taq* DNA Polymerase protocol (Promega Madison, Wis). The PCR conditions were as follow: 10 min of initial denaturation at 94°C and 30 cycles at 94°C for 1 min and annealing at 52/42°C for 1 min and 72°C for 2 min, followed by 10 min of final primer extension. DGGE analysis was carried out using a D-Code Universal Detection System instrument (Bio-Rad) and a model 475 gradient former according to the manufacturer's instructions (Bio-Rad). Polyacrylamide (6%; 37.5:1 acrylamide-bisacrylamide) gels with a 30 to 60% urea-formamide denaturant gradient (100% urea-formamide contains 7 M urea and 40% deionized formamide) were used in 1x TAE (Tris-acetate-EDTA) buffer, pH 7.4, at 200 V for 4 h at 60°C. Gels were stained with ethidium bromide and visualized under UV illumination. About

100 bands were cut from the gel with a sterile blade and placed in sterile vials with 100 µL of Milli-Q water. DNA was allowed to diffuse into the water at 4 °C overnight. Five microliters of the eluate were used as a DNA template in a PCR of 50 µL with the primers described above but without the GC clamp. A total of 51 bands (30 for *Bacteria* and 21 for *Archaea*) yielded sequences that were identified by using the Ribosomal Database Project and the ARB software package (34).

Clone library construction.

The 16S rRNA genes were amplified with the primers sets 27F-1492R (T_a = 57°C) for *Bacteria* and 25F-1492R (T_a = 52°C) for *Archaea*. The PCR conditions were as follow: 10 min of initial denaturation at 94°C and 30 cycles at 94°C for 1 min and annealing temperature at 57/52°C for 1 min and 72°C for 2 min, followed by 10 min of final primer extension. PCR products were gel purified with the GFX™ PCR DNA and gel band purification kit (GE Healthcare) and cloned in *Escherichia coli* DH5α competent cells by using the pGEM-T Easy vector (Promega, Madison, WI) according to the manufacturer's instructions. Recombinant clones were identified as white colonies on chromogenic indicator plates. Isolated clones were grown overnight in 1.5 mL of broth medium containing 100 mg of ampicillin per liter. Recombinant plasmids were retrieved by an alkaline lysis protocol (4), and the correct size of inserts was verified by gel electrophoresis. Plasmid DNA of positive clones were screened by Amplified rRNA gene restriction analysis (ARDRA) using endonuclease BfuCI (1 U, 4 h, 37°C) and grouped according to the restriction patterns obtained. Two members of each group were then sequenced using a Big-Dye sequencing kit (Applied Biosystems) following the manufacturer´s instructions.

Sequences were assembled using the DNABaser program, and prior to phylogenetic analysis, vector sequences flanking the 16S rRNA gene inserts were removed. Sequences were compared with those in NCBI databases using the Ribosomal Database Project (35) to identify the closest sequences. Clone sequences were checked for chimeras using the program Chimera Check from green-genes (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi). Complete sequences (>1400 bp) obtained in this study were added to a database of over 50000 homologous prokaryotic 16S rRNA primary structures by using the alignment tool of the ARB software package. Phylogenetic reconstruction was performed by using the three algorithms implemented in the ARB package (34). A consensus tree was generated, and bootstrap analysis was performed.

An ARB package-generated distance matrix was used to assign sequences to operational taxonomic units (OTUs). The coverage of the clone libraries was calculated using the equation described by Hansen and Singleton (21), and the sampling efficiency in the clone libraries was assessed using the Analytic Rarefaction (v 1.3) software (http://www.uga.edu/strata/software/) originally described by Heck *et al*. (23). PAST software (v 1.82b) (20) was used to compute the statistical indexes for the archaeal and bacterial sequences in each sampling station.

Nucleotide sequence accession numbers.

The 16SrRNA genes sequences determined in this study have been deposited in the GenBank database under accession numbers. HQ730609-HQ730756; HQ853235-HQ853236; HQ916664-HQ916667, combining sequence and contextual data in compliance with the Genomic Standars Consortium (GSC) with the CDinFusion tool (51).

RESULTS

Physicochemical parameters of the sampling site

Significant differences between both sample sites (*JL* and SN) were observed (Table 1).

Table 1: Physicochemical parameters of both sample sites over two years.

	year 2008			year 2009		
Sample	Depth (cm)	pH	Redox (mV)	Depth (cm)	pH	Redox (mV)
JL1	8	3.8	168	7	3.8	200
JL2	11	3.9	69	12	4.6	-13
JL3	14	4	113	16	4	53
JL4	26	4.4	8	22	4.1	12
JL5	30	5.3	-120	26	4.4	1
JL6	33	4.9	-27	33	5.7	-132
JL7	38	5.4	-71	40	4.7	-30
SN1	21-26	3.9	200	25	2.3	255
SN2	40-45	4	-13	43	3.63	47

JL cores were banded, showed local variations from oxidizing zones to reducing zones with a redox range from 200 to -120 and pH range from 3.9 to 5.7. Meanwhile, the SN sampling site comprised only two layers and showed a more oxidized zone in the superficial layer defined by the occurrence of brownish Fe(III) (hydr)oxides, a positive redox potential (+200mV), a low pH of 2 to 4, and a deeper layer with less oxidant or even reducing redox potential.

DGGE fingerprint analyses

Both sites were analysed at different depths to evaluate the level of diversity and to elucidate the possible correlation between the distribution of microorganisms with the physicochemical parameters. The bacterial fingerprint (Fig. 1A) showed a relatively high number of bands compared to the low number found in previous water column studies (18).

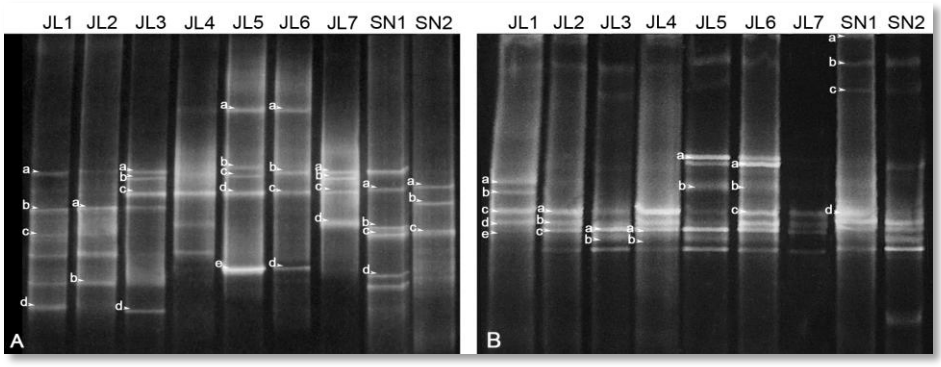


Fig. 1: DGGE fingerprints of 16S rRNA obtained with domain-specific primers for *Bacteria* (A) and *Archaea* (B). Lane names are shown according to depth and sample site.

The pattern of bands differed between the two sampling sites and, on a global perspective, between depths. *Bacteria* sequences clustered in the phyla *Proteobacteria* (classes *Gammaproteobacteria* and *Deltaproteobacteria*), *Bacteroidetes, Acidobacteria* and *Firmicutes.* As seen in Fig. 2, there is a correlation between layers and the phylogenetic affiliation of the sequences.

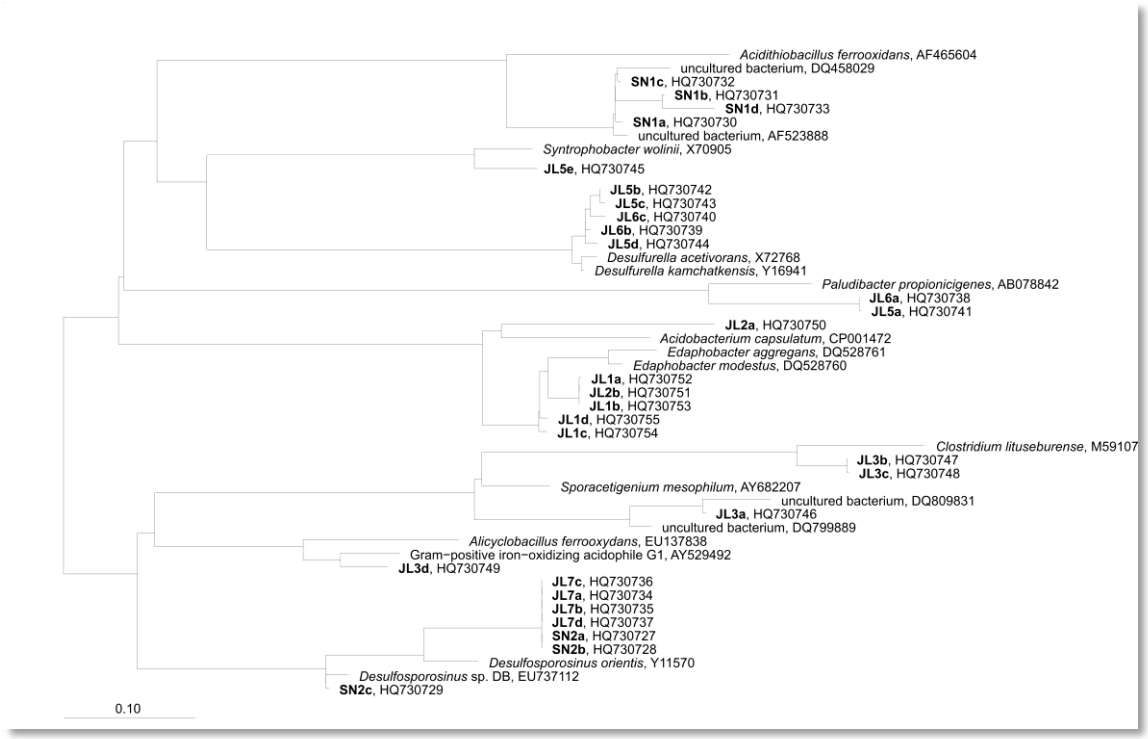


Fig. 2: Phylogenetic affiliation of bacterial DGGE sequences obtained from the Tinto River samples. The phylogenetic tree was generated using parsimony with the ARB program. The bar indicates a 10% estimated sequences divergence. The sequences from Tinto River are indicated in bold type, and designations after the organism names or identifiers are GenBank accession numbers

In the surface layers of *JL* dam (*JL1* and *JL2*), sequences belong to the phylum *Acidobacteria*, which includes acidophilic bacteria typically found in soils. In the next layer (*JL3*) sequences belong to the phylum *Firmicutes*, clustering in the order *Clostridiales* (*Sporacetigenium* spp.) and *Bacillales* (*Alicyclobacillus* spp). In the deepest layers (*JL5*, *JL6*, *JL7*), extremely anaerobic organisms were found, including sulfate-reducing bacteria, such as *Desulfurella* and *Desulfosporosinus* spp., and *Syntrophobacter spp*, propionate-degrading syntrophic bacteria typically from neutral pH environments, as well as *Paludibacter* spp., a fermenting *Bacteroidetes*. In the surface layer of *SN* dam (*SN1*), sequences were identified as belonging to the family *Acidithiobacillaceae* (*Gammaproteobacteria* class). In the deeper layer (*SN2*), organisms were related to the spore-forming sulfate-reducing bacteria *Desulfosporosinus* spp.

The archaeal fingerprints (Fig.1B) had a lower number of bands than the *Bacteria* and their phylogenetic distribution was less varied; in fact, all the *Archaea* sequences cluster in the phylum *Euryarchaeota*, in the order *Thermoplasmatales,* independently of the sample site and depth (Fig. 3).

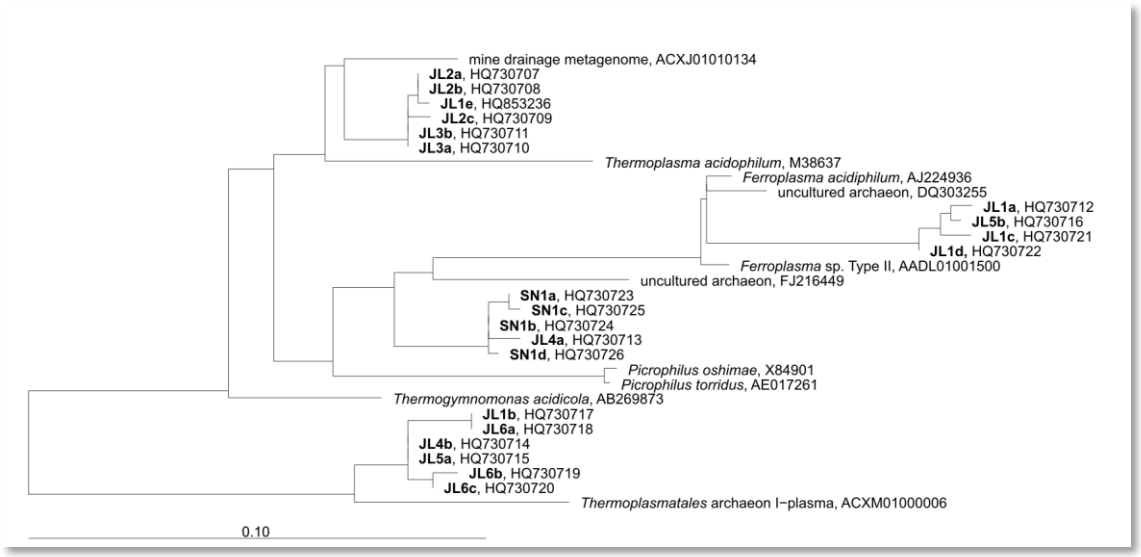


Fig. 3: Phylogenetic affiliation of archaeal DGGE sequences obtained from the Tinto River samples. The phylogenetic tree was generated using parsimony with the ARB program. The bar indicates a 10% estimated sequences divergence. The sequences from Tinto River are indicated in bold type, and designations after the organism names or identifiers are GenBank accession numbers.

Bacterial clone libraries diversity

A total of 489 sequences (>1400 bp) were grouped into 44 phylotypes or OTUs on the basis 97% sequence similarity (42). The taxonomic affiliation of the cloned sequences revealed a high degree of biodiversity. The microorganisms fell into four different phyla with at least 20 different genera. Although PCR-based methods are not quantitative, *Proteobacteria* and *Firmicutes* were the most represented phyla in our study, each comprising of 17 phylotypes (38.6%), followed by the *Acidobacteria* and *Actinobacteria* with 5 OTUs each (11.4%).

(i) Proteobacteria (Fig. 4).

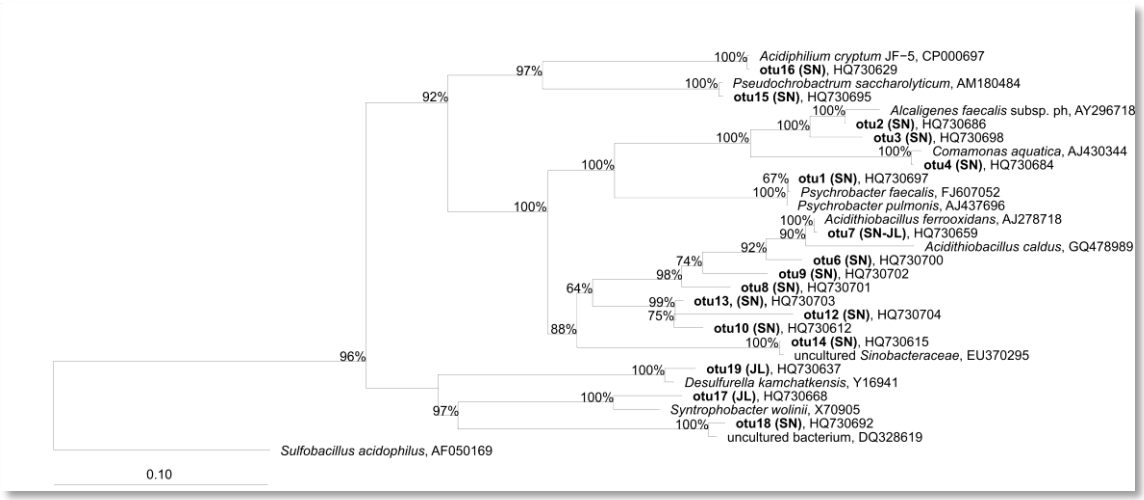


Fig. 4: Phylogenetic affiliation of 16S rRNA sequences of *Proteobacteria* phylum obtained from Tinto River samples. A consensus phylogenetic tree was generated using parsimony, neighbour-joining and maximum likehood analysis with different set of filters, showing them a stable branching. 100 bootstrap replicates were performed. The bar indicates a 10% estimated sequences divergence. The sequences from Tinto River are indicated in bold type, and the designations after the organism names or identifiers are GenBank accession numbers.

(a) Alphaproteobacteria: OTU 16 was affiliated with *Acidiphilium cryptum,* a heterotrophic species which is widely distributed in bioleaching and AMD enviroments and capable of respiring ferric to ferrous iron by oxidizing reduced carbon compounds (25). OTU 15 is related to *Pseudochrobactrum asaccharolyticum*, a denitrifying bacteria capable of fermentative metabolism (28). ***(b) Betaproteobacteria***. OTUs 2 and 3 were assigned to the genus *Alcaligenes,* with OTU 2 identified as *Alcaligenes faecalis.* Most of the strains are capable of anaerobic respiration with nitrite, but not nitrate, as a sole electron acceptor (40). OTU 4 was affiliated with *Comamonas aquaticus,* which has a respiratory metabolism using oxygen as the terminal acceptor and is capable of nitrate-reduction (49). ***(c) Gammaproteobacteria*** OTUs 6, 7, 8, 9, 11, and 13 clustered in the order *Acidithiobacillales,* of which OTUs 6-9 were affiliated with the genus *Acidithiobacillus,* OTU 7 was identified as *At. ferrooxidans*, one of the most well studied bioleaching bacteria, which derives energy mainly from the oxidation of reduced sulfur compounds and/or ferrous iron. Clones belonging to OTUs 10, 11 and 13 clustered into the *Acidithiobacillales* group RCP1-48, which also has clones isolated from AMD systems. OTU 14 (uncultured *Sinobacteraceae*) could not be assigned to any described species but showed high similarity to uncultured microorganisms retrieved from acid environments, including the Tinto River like clone EU370296. OTU 1 was affiliated with the genus *Psychrobacter*, which is related with clones isolated from sediments. ***(d) Deltaproteobacteria*** OTU 17 was assigned to the genus *Syntrophobacter* and OTU 19 was identified as *Desulfurella,* both of which are implicated in the sulfur cycle. Members of the genus *Syntrophobacter* can use sulfate as an electron acceptor and oxidize propionate via the methylmalonyl coenzyme A pathway, and *Desulfurella* spp. are acid tolerant sulfur-reducing bacteria detected in several acid environments as well as in AMD remediation bioreactors (27). OTU 18 clustered with uncultured *Bacteriovoraceae* bacterium related to clones of the organism with GenBank accession number DQ328619 obtained from an extreme acid mine drainage sediment. *Deltaproteobacteria* were detected for the first time in AMD systems at the Richmond Mine (7), and since then they have been detected in some similar systems (8, 9, 16, 22, 48, 50).

***(ii) Firmicutes* (Fig. 5)**

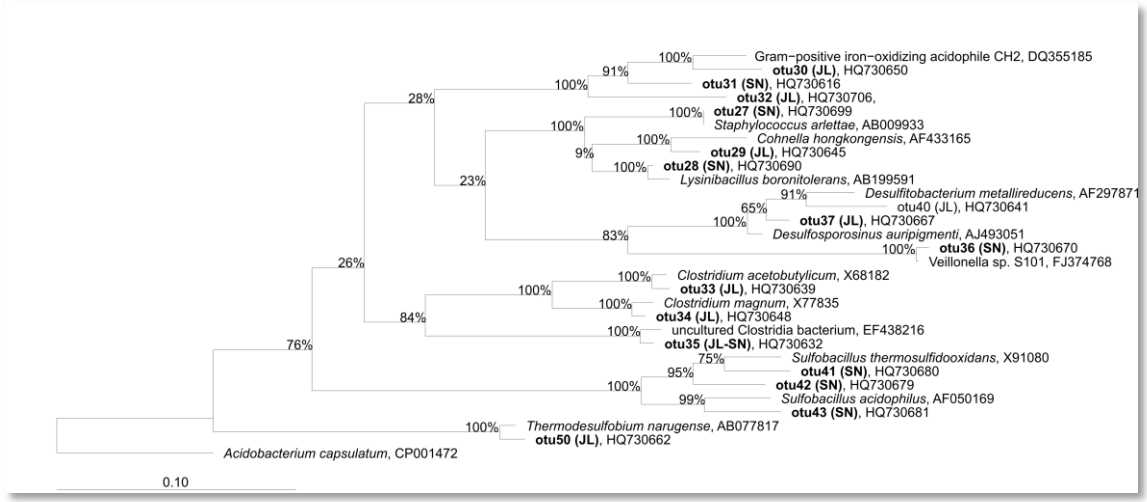


Fig. 5: Phylogenetic affiliation of 16S rRNA sequences of Firmicutes phylum obtained from Tinto River samples. A consensus phylogenetic tree was generated using parsimony, neighbour-joining and maximum likehood analysis with different set of filters, showing them a stable branching. 100 bootstrap replicates were performed. The bar indicates a 10% estimated sequences divergence. The sequences from Tinto River are indicated in bold type, and the designations after the organism names or identifiers are GenBank accession numbers..

The sequenced clones of the *Firmicutes* were distributed evenly over the two relevant environmental subgroups, classes *Bacilli* and *Clostridia*. All the OTUs could be identified at least at the genus level as *Staphylococcus* (OTU 27), *Lysinibacillus* (OTU 28), *Cohnella* (OTU 29), *Alicyclobacillus* (OTUs 30 to 32), *Clostridium* (OTU 33 to 35), *Veillonella* (OTU 36), *Desulfosporosinus* (OTU 37), *Desulfitobacterium* (OTU 39-40), *Sulfobacillus* (OTU 41 to 43), and *Thermodesulfobium narugense* (OTU 50). Several of these microorganisms are involved in the sulfur cycle. *Sulfobacillus* spp. are iron- and sulfur- oxidizers and grow over a broad range of temperature (20 to 60°C). *Desulfosporosinus* spp. are obligately anaerobic, sulfate-reducing bacteria with the ability to sporulate, making them more resistant to acidic conditions (15). The majority of these clone sequences were 97% similar to *Desulfosporosinus orientis*, an organism that has previously been detected in other acidic environments, including acidic mining-impacted lake sediments (31). *Desulfitobacterium* spp. are very versatile microorganism that can use a wide variety of electron acceptors, such as nitrate, sulfite, metals, humic acids, and halogenated organic compounds (48). *Thermodesulfobium* is a moderate thermophilic autotroph that is able to grow on H_2/CO_2 by sulfate- respiration (36).

(iii) Acidobacteria: Seven OTUs were associated with the *Acidobacteria*. OTUs 20 to 25 clustered in *Acidobacterium* group and OTU 26 was related to the genus *Edaphobacter*. Although most of the clones were situated at considerable distances from the cultured members of this phylum, OTU 24 was identified as *Acidobacterium capsulatum,* a bacterium that formed Fe(II) under anoxic conditions at pHs ranging from 3 to 5 in association with the anaerobic respiration of glucose (12).

(iv) Actinobacteria: All clones comprising OTUs 45 to 49 clustered into the family *Acidimicrobiaceae,* and although they could not be assigned to described species, they showed high similarity to uncultured microorganisms retrieved from AMD and a low similarity (90%) to *Acidimicrobium ferrooxidans,* a moderately thermophilic and acidophilic bacterium with a versatile metabolism (11): it oxidizes ferrous-iron or reduces ferric-iron as *At. ferrooxidans*.

Archaeal clone (*Euryarchaeota)* library diversity

After chimeras and low-quality sequences were removed, a total of 78 archaeal clones distributed in 7 OTUs were retrieved. The clones retrieved from both sampling stations were phylogenetically homogeneous and belonged to the order *Thermoplasmatales*. Although *SN* clones could not be identified as belonging to any cultured species, *JL* clones belonged to the genus *Thermoplasma*, a moderate thermoacidophilic facultatively anaerobic archaeon which respires sulfur with organic carbon as an electron donor.

Relative distribution of the major phylogenetic groups in both sample sites.

The distribution of the major phylogenetic groups differed between sample sites (Fig. 6). Just 2 of 44 OTUs were shared between them. In *JL* dam, a total of 249 sequences (>1400 bp) were grouped into 18 OTUs. In *SN* dam, a total of 240 sequences were grouped into 28 OTUs. In *JL* dam, the group of *Bacteria* detected with the most numerous organisms was the phylum *Firmicutes* (56.6%), followed by phylum *Acidobacteria* (27.3%) and the class *Deltaproteobacteria* (11.6%). Organisms of the *Actinobacteria* phylum (4%) and *Gammaproteobacteria* class (0.4%) were less abundant. In *SN* dam, the phylum *Proteobacteria* (72.1%) was the most represented, mainly falling into the *Alphaproteobacteria* (39.6%) and *Gammaproteobacteria* (30.4%), followed

by *Actinobacteria* (20.4%). Organisms of the phyla *Firmicutes* (5.3%) and *Acidobacteria* (1.7%) were present in low percentages.

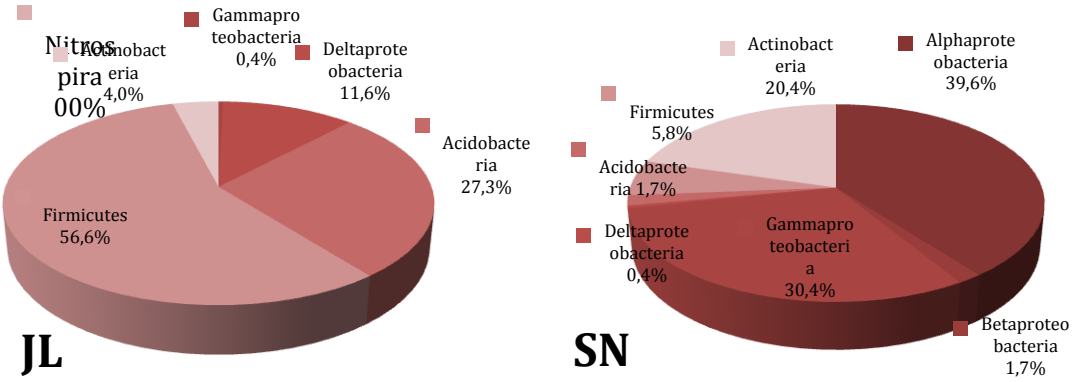


Fig. 6: Phylogenetic composition of the bacterial 16S rRNA gene libraries of the two sample sites JL dam (A) and SN dam (B).

Diversity indexes, coverage and rarefaction analyses of the 16S rRNA gene libraries.

Rarefaction curves (Fig. 7) and Good's coverage values and diversity indexes (Table 2) were calculated separately for *Archaea* and *Bacteria* in each sample site (*SN* and *JL*).

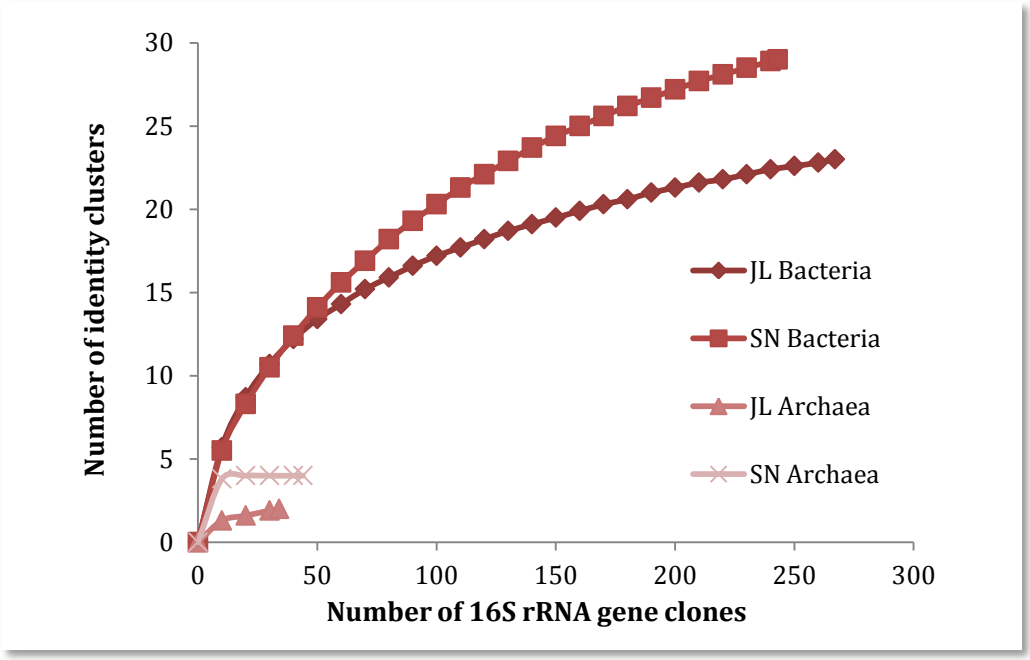


Fig. 7: Rarefaction curves obtained for the sequences of the 16S rRNA gene libraries for *Bacteria* and *Archaea* of both sampling sites.

Good's value, an estimation of the proportion of the population represented by the retrieved sequences (19), indicates high coverage in the archaeal and bacterial libraries of both sample sites, in agreement with the rarefaction curves. Dominance (D) values are similar in both samples sites but differ between the two domains. Whereas in *Bacteria* this value is close to 0, indicating that no OTUs predominate in the community, in *Archaea, D* values are higher, indicating the predominance of some OTUs in the sequence data set. In accordance, the Shannon-Wiever index value was lower.

Table 2: Statistical indexes for bacterial and archaeal sequences in each sampling site using PAST software.

	Bacteria		*Archaea*	
Station	JL	SN	JL	SN
Number of sequences	249	240	34	44
Number of OTU´s	18	28	2	4
Good´s coverage value	98.4%	96.3%	97.1%	100%
Shannon-Weiner index	2.01	2.20	0.13	0.25
Dominance-D value	0.22	0.19	0.94	0.87

DISCUSION

Due to the important biotechnological, ecological and astrobiological implications of AMD systems, these environments have been studied intensively during the last decade (3, 16, 43, 47). In addition, a useful review of carbon, iron and sulfur metabolism in acidophilic microorganisms has been recently published (26). However, fewer studies have been performed on the anaerobic zones of these environments (5, 22), and some have focused exclusively on Fe-cycling bacteria (33) or methanogens (46).

DGGE fingerprint analysis of Tinto River sediments showed a good correlation between the physicochemical parameters of the different layers and the microorganisms inhabiting them. In *JL* dam, the local variations within layers provide high physicochemical heterogeneity, and their influence can be stronger than that of depth. Despite this fact, a general tendency can be observed, with the layers varying from oxidizing and acidic conditions in the upper parts toward strongly reductant and slighty acidic ones in the deepest layers. Consequently, the dominant microorganisms shifted from *Acidobacteria* in the upper parts to fermentative organisms like *Paludibacter* spp. and *Clostridium* spp. when the conditions became less oxidizing and to sulfate-reducing bacteria like *Desulfurella* spp. and *Desulfosporosinus* spp. when the conditions were strongly reducing. A similar distribution was found in *SN* dam, with variation of microorganisms from those related to the *Acidithiobacillaceae* family under oxidizing conditions to *Desulfosporosinus* spp under reducing ones.

The phylogenetic analyses of 16S rRNA genes retrieved from the Tinto River sediments identified bacterial sequences from the *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Acidobacteria* phyla, and the archaeal sequences from the *Euryarchaeota/ Thermoplasmatales* group. Despite some similarities to sequences obtained in previous studies of Tinto River and other AMD systems (16, 17, 33, 43), some important differences could be identified. For example, no sequences related to *Leptospirillum* were found. This lithoautotrophic Fe(II) oxidizer has been shown to dominate microbial communities in extremely acidic AMD environments (41). Its preference to grow at a lower pH than *At. ferrooxidans* and its strict aerobic metabolism explain why it did not appear in Tinto river sediments, leaving the role of iron-oxidizer in the oxidizing part of these moderate acidophilic sediments to *Ferroplasma* spp., *At. ferrooxidans, Sulfobacillus* spp. and *Alicyclobacillus* spp. in the anoxic parts (33).

Sulfate reduction and methanogenesis are usually inhibited in the presence of more electro-positive electron acceptors as ferric iron (6, 10), which become the main electron acceptor for the oxidation of organic matter in anoxic AMD environments. So, the Fe(III)-reducing bacteria *Acidiphilium* spp., *Acidobacterium* spp., and *Sulfobacillus* spp. appear widely in the clones

libraries. Some 27.3% of the bacteria were identified as *Acidobacteria* and were specifically related to *A. capsulatum*. With increasing pH, the solubility of iron decreases and sulfate reduction seems to play a more important role. Almost 20% of the sequences retrieved in *JL* dam belonged to sulfate-reducing bacterium-related microorganisms: *Syntrophobacter* spp., *Desulfosporosinus* spp., *Desulfurella* spp., *Desulfitobacterium spp.* and *Thermodesulfobium* spp. Their prevalence should not be surprising, because in presence of high concentration of sulfate, as the case of Tinto River, sulfate reducers dominate over methane producers when competing with each other over limited resources. Methanogens likely do not play a significant role in the ecology of the sediments. In fact, we failed to identify them by molecular approaches. However, their presence must not be ruled out: methane production was observed *in situ* and in the laboratory (data not shown). In addition, the occurrence of methanogens in *JL* dam sediments has recently been described (46). At *SN* dam, with a comparatively low pH, Fe(III) reducers *Acidiphilium* spp., *Sulfobacillus* spp. and *At. ferrooxidans* predominated.

From our data, other anaerobic metabolisms apart from iron and sulfate reduction can be inferred. Several sequences related to potential nitrate or nitrite reducers such as *Alcaligenes faecalis, Pseudochrobactrum* spp., *Pseudomonas* spp., and *Bacillus* spp. were found. The concentrations of both nitrate and nitrite in the pore water of the sediments were below the detection limit (1 ppb), so nitrogen must be a limiting factor for bacterial growth in this environment and any input will be removed quickly. In previous studies of the boreholes in the Tinto River (2), the fluid extracted from those underground habitats contained dissolved gases, such as H_2, N_2O and CH_4. Although the chemical origin of H_2 was proposed to be by a water/rock interaction, the identification of H_2-producing bacteria, such as *Syntrophobacter* spp. or *Clostridium* spp., introduces the possibility of a biological production of this gas.

In an extreme acidic, oxidizing, and cold bulk environment, we have found zones with slightly acidic pH and reducing redox potential. In accordance with this finding, sequences corresponding to microorganisms with forms of metabolism (such as sulfate and nitrate/nitrite reduction) that do not normally take place under the physicochemical conditions prevailing in the Tinto River were retrieved. Protection from acidity (29) and even the removal of oxygen by active respiration (30) have been described. In a similar way, the exothermic microbial oxidation of pyrite, which releases heat under both aerobic and anaerobic conditions, could be the source of microenvironments with relatively high temperatures, explaining the presence of moderate thermophilic organisms (*Alicyclobacillus* spp., *Cohnella* spp., *Desulfurella* spp., *Thermodesulfobium* spp., *Sulfobacillus* spp., and *Thermoplasma* spp.) found in the sediments.

Geomicrobiologial model of the sediments of Tinto River

In this study, Tinto sediments were analysed by combining physicochemical data with molecular ecology studies. Rarefaction curves and Good´s coverage values showed that almost all of the diversity was covered with our data set. So, through integration of geochemical and biological information, a model of the geomicrobiological processes that allow organisms to thrive in this extreme environment is suggested (Fig. 8).

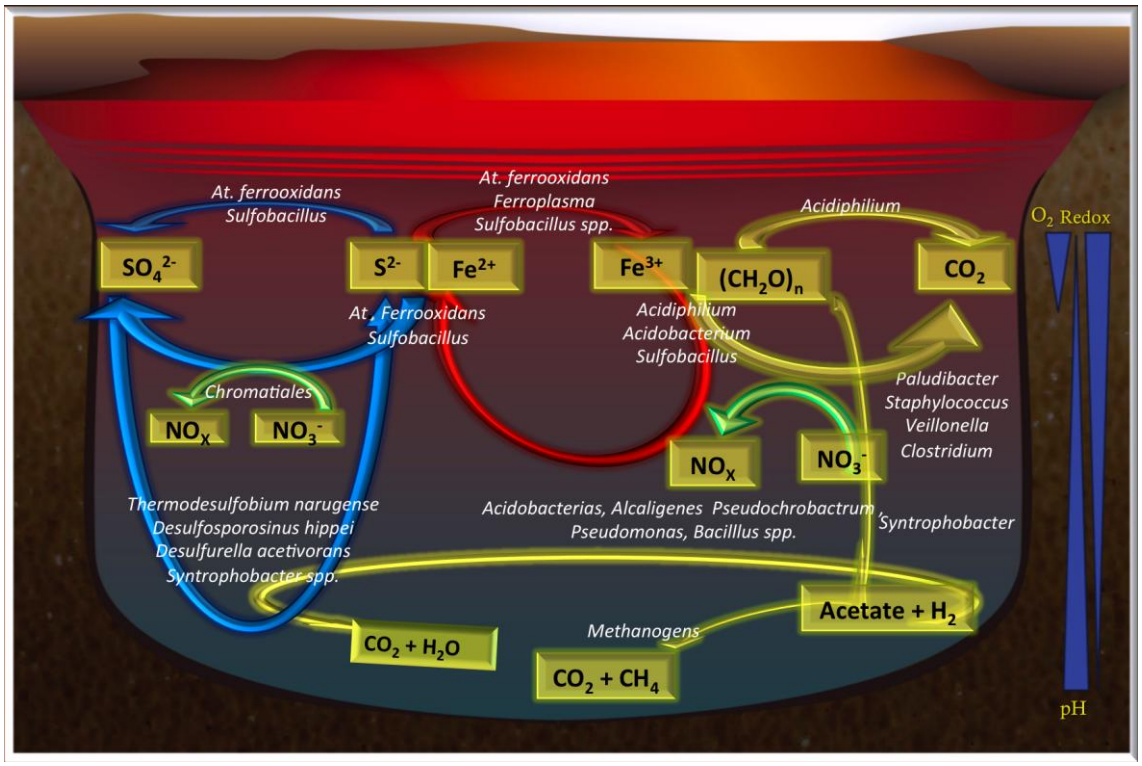


Fig. 8: Geomicrobiological model of Tinto River sediments, an AMD-related environment. Iron, sulfur and carbon cycles are associated to the microorganisms involved in them.

In contrast to previous studies on the water column, the molecular ecology of Tinto River sediments is not based exclusively on the iron cycle. In fact, it seems that the sulfur cycle and the removal of organic matter are predominant in this particular system. On the basis of the mineralogical composition of the Iberian Pyritic Belt, the main chemolithotrophic substrate available would be pyrite (FeS_2), and that is the basis for the development of a complete community of microorganisms.

Iron in its reduced form, Fe(II), would be the main energy source of the autotrophic, facultatively anaerobic bacterium *At. ferrooxidans,* as well as members of the versatile genera *Sulfobacillus* spp. and *Alicyclobacillus* spp. and the archaeon *Ferroplasma* spp. As AMD solutions are iron rich because of the high solubility of iron at low pH, Fe(III) concentrations can exceed oxygen concentrations by several orders of magnitude even in surface layers, so Fe(III) can be widely used as electron acceptor in microbial metabolism (12, 13). Thus, Fe(III) can be reduced, coupled to the oxidation of organic matter by heterotrophic acidophiles such as *Acidiphilium* spp., *Sulfobacillus* spp., *Acidobacterium* spp. or coupled to the oxidation of S^0 under anoxic conditions by *At. ferrooxidans* and *Sulfobacillus* spp. These processes imply the regeneration of the ferrous iron as an energy source, completing the iron cycle with the consortia of microorganism thriving in these sediments.

The second crucial metabolites in this system are sulfur compounds. Sulfide is oxidized to sulfate by the chemolitotrophic bacterium *At. ferrooxidans* under both aerobic and anaerobic conditions and is coupled to Fe(III) reduction in the latter case. Sulfate reduction is carried out by bacteria such as *Desulfosporosinus* spp., *Desulfurella* spp., *Thermodesulfobium* spp., and *Syntrophobacter* spp. The presence of sulfate-reducing bacteria in the Tinto River system is evidence of microbial sulfate reduction at low pH, completing the sulfur cycle in Tinto sediments.

Regarding the carbon cycle, both fermentative and respiring types of metabolisms are present. There is a fermentative pathway that starts with organisms such as *Paludibacter, Veillonella, Staphylococcus,* and *Clostridium* spp. This creates a complex mix of intermediates, and some of them, e.g., propionate, can be used by the syntrophic bacteria *Syntrophobacter* spp., which generates hydrogen and acetate. These compounds act as substrates for the metabolism of sulfate-reducing bacteria and methanogens. Ferric iron can be respired by heterotrophic bacteria of the genera *Acidiphilium*, *Sulfobacillus*, and *Acidobacterium*, which oxidize organic matter, using it as electron acceptor. Although their metabolism is not predominant, there are enough data to support the presence of methanogens (33, 46) that would complete the anaerobic degradation.

Our study implies that organic matter degradation must also be coupled to nitrate reduction mediated by organisms such as *Alcaligenes faecalis, Pseudochrobactrum* spp., *Pseudomonas* spp., and *Bacillus* spp. In fact, nitrous oxides have been measured in bioaugmented cultures of Tinto River sediments (data not shown).

In conclusion, the identification of microorganisms in this study and a detailed examination of their corresponding metabolism have allowed us to compile a model of the iron, sulfur and carbon cycles and their relation to microbial ecology in AMD-related sediments.

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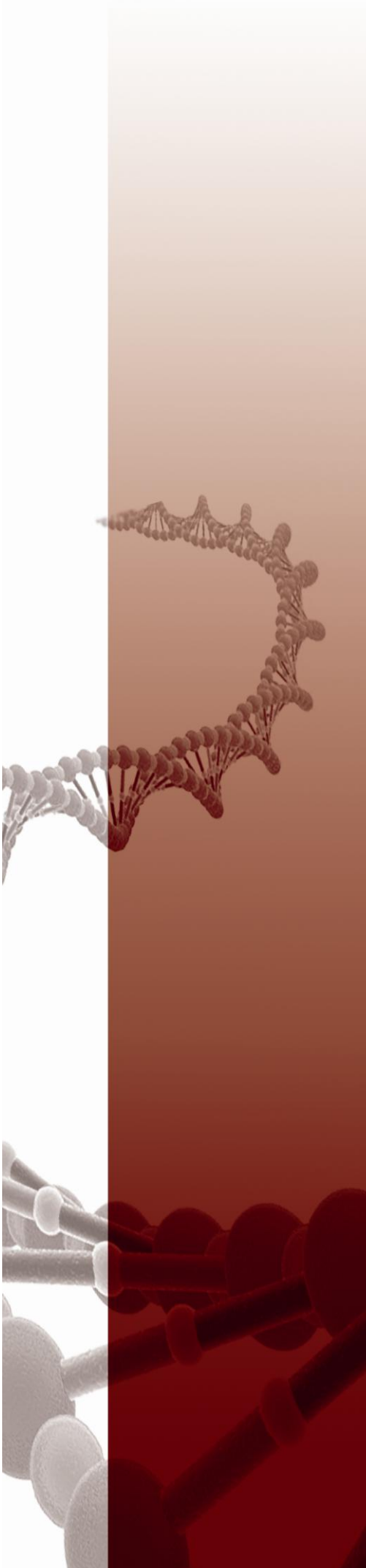
CHAPTER 2: Quantification of Tinto River sediment microbial communities: the importance of Sulfate-Reducing Bacteria and their role in attenuating acid mine drainage



INTRODUCTION

The Tinto River is a natural acidic rock drainage (ARD) environment located in the Huelva region, in southwestern Spain. On its way from Peña de Hierro to the Atlantic Ocean, the Tinto River emerges from the core of the Iberian Pyritic Belt (IPB), one of the largest metal sulfides (pyrite, chalcopyrite, etc.) deposit on Earth. When exposed to air and water, the microbiologically enhanced oxidation of sulfides produces waters with low pH (pH 2.3) and high ferric iron (~2 g L^{-1}) and sulfate (~6 g L^{-1}) content along the river course. Low pH and ferric iron facilitate metal solubilization; therefore, when these waters come into contact with metallic sulfides, they become highly metalliferous (Cu: ~0.1 g L^{-1}; Zn: ~0.2 g L^{-1}, etc.) (20).

ARD results in contamination of aquifer with secondary effects like the death of fish, rodents, livestock, and reduced crop yields (36). These unpleasant consequences have led to efforts to remediate ARD environments (21). Besides the environmental concerns, there are biological, geochemical, and biotechnological reasons to analyze microbial populations in acidic environments and to understand their ecology. In ARD, acidophilic, chemolithotrophic, aerobic *Bacteria* and *Archaea* dissolve metallic sulfides by oxidizing the iron and sulfur components. Products resulting from these oxidation processes can be used as electron acceptors in dissimilatory reduction of ferric iron and sulfate under anaerobic conditions linked to the carbon and nitrogen cycles. So far, only ARD aquifers have been studied intensively (7, 8, 19, 22, 23); in contrast, little is known about the ARD sediments (17, 41).

At Tinto River, knowledge of the diversity and abundance of benthic microbial populations is limited to a methanogen population study (43) and a description of the microbial ecology of two contrasting sampling sites (SN and JL dams) (42). In the latter study, an extensive survey of the Tinto River sediment microbiota was performed using two culture independent approaches: denaturing gradient gel electrophoresis and 16S rRNA gene sequencing. The taxonomic affiliation of the identified *Bacteria* showed a high degree of diversity, falling into 5 different phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Acidobacteria*, and *Actinobacteria*; meanwhile, all the *Archaea* were affiliated with the order *Thermoplasmatales*. Microorganisms involved in the iron (*Acidithiobacillus ferrooxidans*, *Sulfobacillus* spp. and *Ferroplasma* spp.), sulfur (*Desulfurella* spp., *Desulfosporosinus* spp. and *Thermodesulfobium* spp.) and carbon (*Acidiphilium* spp., *Bacillus* spp., *Clostridium* spp. and *Acidobacterium* spp.) cycles were identified. However, the abundance of these groups of organisms was unknown. In this study, the microbial communities of the Tinto River sediments were quantified for the first time, and analyzed in relation to the physico-chemical properties of the sediments with the objective of identifying the dominant and active organisms thriving in the sediments and inferring the main microbial activities taking place.

MATERIALS AND METHODS

Field site description and sampling

The selected sediments are located in the Tinto River basin (Huelva). Samples were collected from two sampling sites (Fig. 1), *SN* dam (37.72173N, 6.557465W) and *JL* dam (37.691207N, 6.560587W) at different positions of the river. *SN* dam is a spring feeding the main stream of

the river, while *JL* is located in the main course of the river. Sediment cores (inner diameter, 7 cm; length, 45 cm) were taken with a sampler (Eijkelkamp Agrisearch equipment, The Netherlands). The cores were sliced at 5 cm intervals. The redox potential (E) and pH of the drill core samples were measured *in situ* with E and pH probes, connected to a Thermo Orion 290A potentiometer placed into the fresh sediment just after extraction of the core. Sediment samples for hybridization were fixed immediately in 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS: 145 mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4 pH 7.6) at 4°C for 4 h, washed twice with PBS then stored in 50% (v/v) ethanol-PBS at -20°C until further use.

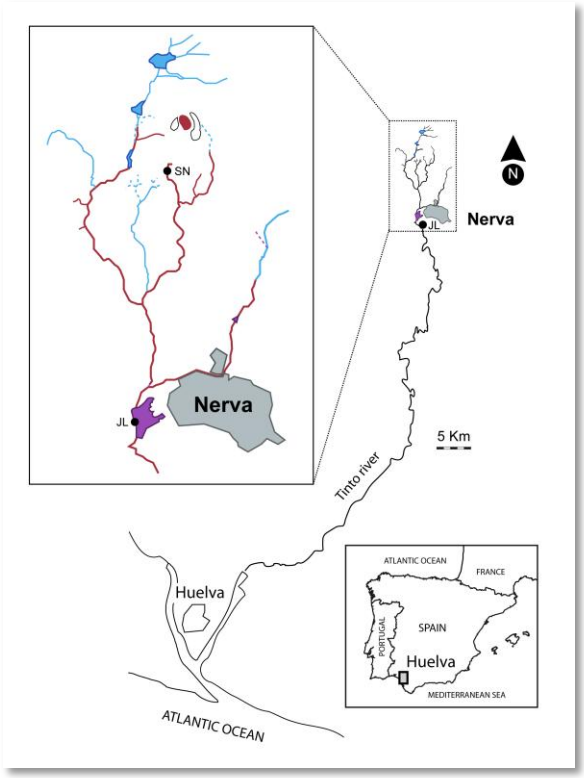


Fig. 1: Map of Tinto River in Huelva (Spain) with the location of the studied dams.

Chemical analysis

According to physico-chemical parameters and visual appearance, the JL dam core was composed of two main layers with black and brown colors, whereas the *SN* core looked homogeneous (Fig. 2a,e). The black (10-15 cm) and brown (25-30 cm) layers from *JL* dam and two layers between 10-15 cm and 25-30 cm from *SN* dam were excised and immediately stored in 15 mL tubes at 4°C until further processing in the laboratory (less than one week). Sediments were centrifuged at 13000 x g for 5 min and the supernatant was analyzed for the presence of heavy metals by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and organic anions/Volatile Fatty Acids (VFA) by Ionic Chromatography (IC) using an 861 Advanced Compact IC instrument. Water samples of both dams were also taken, kept at 4°C and analyzed by Total Reflection X-ray Fluorescence (TXRF) for S, P, Mn, Fe, Cu, Zn, As and Ca; by ICP-MS for the rest of the elements and by thermo-chemical oxidation (Thermostar LT200 of Hachlange) for total organic carbon (TOC).

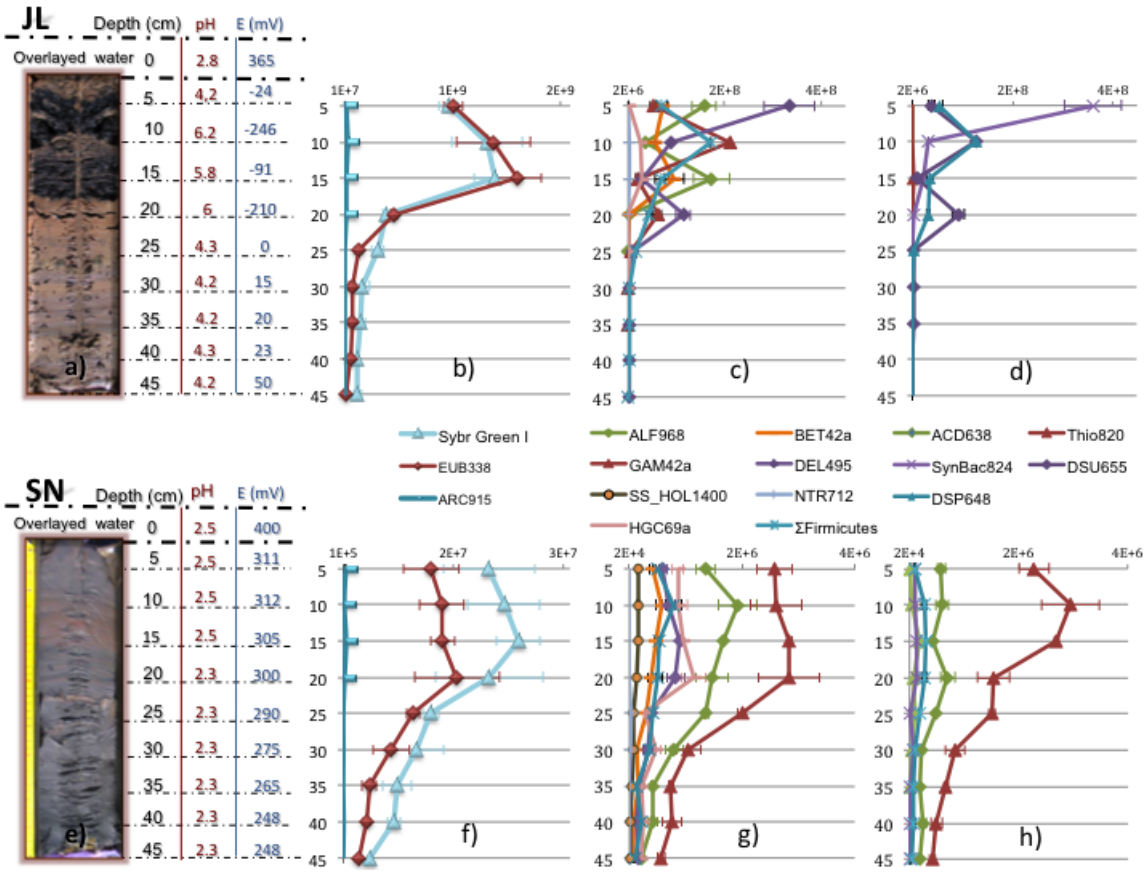


Fig. 2: Depth profiles of pH and redox *JL* (a) and *SN* (e), Sybr Green I and Domain-specific probes counts *JL* (b) and *SN* (f), phylum-specific probes counts *JL* (c) and *SN* (g) and genera-specific probes counts *JL* (d) and *SN* (h).

Quantification of microorganisms

(i) Sybr green I direct counts.

Aliquots of fixed samples were sonicated to detach bacteria from the particles with a sterilized Sonic probe (Bandelin, Sonopuls HD200) for 20 sec, 3 times at 20% intensity. Samples were kept on ice for 4 min to precipitate large sediment particles that could clog the filter and then the supernatant was gently filtered through a black 0.2 μm polycarbonate membrane (Nuclepore, 25 mm diameter) (<0.2 bar). The filters were stained with SybrGreen I in Moviol Medium as described elsewhere (30). Samples were examined with an epifluorescence microscope (Axioplan 2, Carl Zeiss, Jena, Germany). A minimum of 1000 SybrGreen I-stained cells or at least 100 independent microscopic fields were counted.

(ii) CARD-FISH.

Samples were filtered following the same procedure as described above for the direct counts, except that white filters (0.2 μm pore size; Millipore GTTP) were used. In situ hybridizations with horseradish peroxidase (HRP)-labeled probes (biomers.net, Ulm, Germany) followed by catalyzed reporter deposition of fluorescently labeled tyramide were carried out as described elsewhere (38). Permeabilization was done with lysozyme (10 mg mL^{-1}) for 90 min at 37°C, followed by achromopeptidase (60 U mL^{-1}) for gram-positive cells (44) and proteinase-K for Archaea (15 μg mL^{-1}) (46). Probe sequences and formamide concentrations required for specific hybridization are given in Table 1.

Table 1: Oligonucleotide probes used in this study.

Probe name	Sequence (5' → 3')	Specificity	[a]FA[%]	References
NON338	ACTCCTACGGGAGGCAGC	-	0	(47)
EUB338	GCTGCCTCCCGTAGGAGT	*Bacteria*	35	(3)
EUB338-II	GCAGCCACCCGTAGGTGT	*Planctomyces*	35	(9)
EUB338-III	GCTGCCACCCGTAGGTGT	*Verrucomicrobiae (and others)*	35	(9)
ALF968	GGTAAGGTTCTGCGCGTT	*Alphaproteobacteria, except of Rickettsiales*	20	(35)
ACD840	CGACACTGAAGTGCTAAGC	*Acidiphilium spp.*	10	(12)
BET42a[b]	GCCTTCCCACTTCGTTT	*Betaproteobacteria*	35	(32)
GAM42a[b]	GCCTTCCCACATCGTTT	*Gammaproteobacteria*	35	(32)
THIO820	ACCAAACATCTAGTATTCATCG	*Acidithiobacillus spp.*	10	(37)
DELTA495a	AGTTAGCCGGTGCTTCCT	*Most Deltaproteobacteria and Gemmatimonadetes*	30	(27)
cDELTA495a	AGTTAGCCGGTGCTTCTT	*Competitor for DEL495a*	-	(31)
DELTA495b	AGT TAG CCG GCG CTTCCT	*Some Deltaproteobacteria*	30	(27)
cDELTA495b	AGTTAGCCGGCGCTTC(T/G)T	*Competitor for DEL495b*	-	(28)
DELTA495c	AATTAGCCGGTGCTTCCT	*Some Deltaproteobacteria*	30	(27)
cDELTA495c	AATTAGCCGGTGCTTCTT	*Competitor for DEL495c*	-	(28)
SYNBAC824	GTACCCGCTACACCTAGT	*Syntrophobacter spp*	10	(4)
DSU655	CGCTTGCTTTTCCCGAAC	*Desulfurella spp.*	35	This study
HGC69a	TATAGTTACCACCGCCGT	*Gram-positive high GC content (Actinobacteria)*	25	(40)
ARCH915	GTGCTCCCCCGCCAATTCCT	*Archaea*	35	(45)
LGC354a	TGGAAGATTCCCTACTGC	*Gram-positive low GC DNA content (Firmicutes)*	35	(33)
LGC354b	CGGAAGATTCCCTACTGC	*Gram-positive low GC DNA content (Firmicutes)*	35	(33)
CLIT135	GTTATCCGTGTGTACAGGG	*Clostridium cluster XI*	0	(15)
CLOST1	TTCTTCCTAATCTCTACGCA	*Clostridia clusters I and II*	30	(24)
NTR712	CGCCTTCGCCACCGGCCTTCC	*Nitrospira group*	35	(10)
NTR712c	CGCCTTCGCCACCGGTGTTCC	*Competitor for NTR712*	-	(10)
DSP648	CTCTCCTGTCCTCAAGAT	*Desulfosporosinus, Desulfitobacterium, Dehalobacter*	30	(18)
SS_HOL1400	TTCGTGATGTGACGGGC	*Acidobacteria*	20	(34)
EUK516	ACCAGACTTGCCCTCC	*Eukarya*	30	(3)

[a]FA [%]: formamide concentration in the hybridization buffer to ensure specific detection of target organisms.
[b]Used with unlabeled GAM42a as competitor. [b]Used with unlabeled BET42a as competitor.

Hybridized samples were examined with an epifluorescence microscope (Axioplan 2). For each probe and sample, roughly 1000 stained cells or at least 100 independent microscopic fields were counted. The signal obtained with probe NON338 – a negative control – at each sampling site was subtracted from the specific accounts. This signal was about 0.2% of the cell counts for each station.

Total cell numbers in core *SN* were too low to allow specific cell counting. Thus, a density gradient centrifugation was carried out to increase the cell density for quantification of less abundant bacterial groups. Cell separation was performed by Histodenz (Sigma-Aldrich) density centrifugation. Subsamples (1 mL) were placed in 2 mL tubes; 1mL of Hystodenz solution (60% w/v in PBS, density 1.3) was carefully placed underneath using a syringe needle to avoid mixing. Centrifugation was performed at 14,000 x g for 90 min at 4°C. The supernatant above the Hystodenz layer was treated as explained above for the rest of the samples. Although there was significant cell loss during the procedure (30% compared to untreated samples), this method was shown to maintain representative ratios between communities (48), thus it could be used for relative counts.

Design, evaluation and application of a *Desulfurella* spp. probe

The high hybridization numbers shown for *Deltaproteobacteria* probe in this study, the abundant presence of *Desulfurella* clones in the former study of Tinto sediments (42) and the lack of a genus-specific probe for *Desulfurella,* made it necessary to design a new probe for this genus. This was done using the probe design tool in the ARB software package (29). The target position of DSU655 probe (655 to 673) was chosen to be in a highly accessible region according to Behrens *et al.* (6) and to be fully specific for the genus *Desulfurella* (checked with

the ARB Probe Match tool). Using the described CARD-FISH protocol, the stringent hybridization conditions of *Desulfurella* probe were determined with positive control cells of *Desulfurella kamchatkensis* and negative control cells of *Syntrophobacter wolinii* (DSM 2805) and *Acidobacterium capsulatum* (DSM 11244) cultures. Hybridization stringency was optimized by increasing formamide concentrations starting with 10% in 5% steps. A 35% formamide concentration was optimal, resulting in high signal intensity of *D. kamchatkensis* and no signal for the negative controls.

Scanning electron microscopy (SEM)

Sediment samples were studied by SEM as described elsewhere (2). Samples were fixed by immersion in glutaraldehyde (2.5%) for 2 h, washed twice in sodium cacodylate buffer (0.2 M, pH 7.1) and dehydrated in a graded series (10, 30, 50, 70, 90, and 100%) of ethanol/water mixtures for 20 min each. After dehydration, the samples were critical point dried and mounted on stubs. After gold shadowing, the samples were examined in a Phillips XL30 EDAX DX4i SEM.

RESULTS

Physico-chemical properties of the sediments

The redox potential and pH values of the sediment profiles were significantly different (p value <0.001 with U Mann Whitney) at the two sites (*JL* and *SN*) (Fig. 2a,b). *JL* cores were banded and varied from reducing (blackish) to oxidizing (brownish) zones with a redox ranging from -246 to 50 mV and a pH range from 6.2 to 4.2, respectively. *SN* cores did not show strong variations with depth, the pH remained between 2.3 to 2.5 and the redox potential between +248 to +312 mV.

Comparing the black and brown layers of *JL* dam, arsenic, heavy metals and sulfate concentrations in the interstitial water were higher in the brown layers. In the black layers, there was a strong decrease (higher than 90%) for Co, Ni, Cu, Zn and As and close to 50% for Fe, Mn and sulfate (Table 2). A concentration of 79 mg L^{-1} of acetate, an intermediary of the anaerobic digestion pathways and a substrate for some methanogens and sulfate-reducers, was measured in the black band, and the concentration in the brown band was as low as 1 mg L^{-1}. On the other hand, there was no stratification with depth at *SN* dam, so the average of both studied layers is present in Table 2.

Table 2a: Heavy metals, metaloids, sulfate and volatile fatty acid concentrations detected in the porewater sediments and percentage of reduction in *JL* layers.

	Concentration (mM)					
Sample	**formate**	**acetate**	**sulfate**	**Fe**	**Co**	**Ni**
SN	$1.1x10^{-2} \pm 2.1x10^{-3}$	$0.1 \pm 2.1 x10^{-2}$	20.4 ± 2.3	92.2 ± 2.1	UDL	UDL
***JL* brown**	$1.3 x10^{-2} \pm 3.2x10^{-3}$	$6.6x10^{-3} \pm 3.2x10^{-4}$	25.5 ± 3.1	100.2 ± 1.2	$1.3x10^{-1} \pm 5x10^{-3}$	$4.4x10^{-2} \pm 8x10^{-4}$
***JL* black**	$1.1x10^{-2} \pm 2.1x10^{-3}$	$1.3 \pm 1.5x10^{-2}$	12.5 ± 1	52.2 ± 3.3	$1.2x10^{-2} \pm 2.1x10^{-3}$	$1x10^{-3} \pm 2.4x10^{-4}$
% Reduction in JL black			51.1	47.9	90.9	97.7

UDL: under detección limit.

Table 2b: Heavy metals, metaloids, sulfate and volatile fatty acid concentrations detected in the porewater sediments and percentage of reduction in *JL* layers.

	Concentration (mM)				
Sample	**Cu**	**Zn**	**As**	**Al**	**Mn**
SN	1.0 ± 0.2	11.2 ± 0.4	$1.1x10^{-2}$ ± $9x10^{-4}$	43 ± 3.5	2.9 ± 0.2
***JL* brown**	$7.9x10^{-3}$ ± $3x10^{-4}$	1.9 ± 0.1	$1.4x10^{-2}$ ± $2.5x10^{-3}$	33± 1.4	4.1 ± 1.4
***JL* black**	$3.1x10^{-4}$ ± $1x10^{-5}$	$6.1x10^{-3}$ ± $4.1x10^{-4}$	$1.3x10^{-3}$ ± $4.5x10^{-3}$	$3.3x10^{-2}$ ± $4x10^{-3}$	2.1 ± 0.1
% Reduction	96.0	99.7	90.6	99.9	48.3

UDL: under detección limit.

Compared to *JL* heavy metal concentrations varied strongly, Co and Ni were under the detection limit while the concentrations of Zn and Cu were much higher.

Chemistries of the overlaying waters at both dams were similar in pH and some metals (Table 3), but differed for K, Mn, Cd, P, S and Fe. S and Fe are the elements that the oxidation of pyrite (the main substrate in the IPB) put into solution in ferric iron and sulfate forms. Both elements were less abundant at *JL* dam, probably due to a dilution along the river of the concentrated ARD waters present at the *SN* dam.

Table 3: Element concentration, Total Organic Carbon (TOC), redox potential and pH values of *SN* and *JL* overlaid waters.

	pH	E (mV)	TOC (mg L^{-1})	Concentration (mM)								
				Mg	**Al**	**Mn**	**Fe**	**Cu**	**Zn**	**As**	**Cd**	**S**
SN	2.5	400	9 ± 1.69	8.25 ± 0.03	14.54 ± 0.7	UDL	41.30 ± 2.4	0.43 ± 0.04	1.19 ± 0.3	0.07 ± $2.1x10^{-3}$	$2.67x10^{-3}$ ± $5x10^{-4}$	85.83 ± 0.4
JL	2.8	365	20 ± 4	12.34 ± 0.07	13.60 ± 0.4	26.07 ± 1.3	16.34 ± 1.7	1.94 ± 0.07	1.41 ± 0.05	0.04 ± $3.7x10^{-3}$	UDL	54.32 ± 0.6

UDL: under detección limit

Total cell counts with the Sybr Green I method

In comparison to DAPI staining, the use of the highly sensitive Sybr Green I dye in combination with the Moviol embedding medium that lowered photo-bleaching and the use of black filters, which have less background, significantly improved the signal to noise ratio. This enabled reliable counting of total cell numbers.

The average of Sybr Green I stained cells of *JL* core (Fig. 2b) was $5.5x10^{8}$ cells g^{-1} wet weight of sediment whereas in *SN* dam sediments (Fig. 2f) the average was $1.4x10^{7}$, which is 40-fold lower. A common feature among depth profiles of both sites is that cell number in both dams is higher in the 15-20 cm layer. After that, depth profiles followed a general trend observed in many sediments of a drastic decrease with depth (25), from $1.4x10^{9}$ cells g^{-1} wet weight in the 15-20 cm layer to $1.3x10^{8}$ in the 25-30 cm layer in *JL* dam (10-fold lower) and from $2.4x10^{7}$ to $3.7x10^{6}$ in *SN* dam (6-fold lower).

Scanning electron microscopy (SEM)

SEM analysis of sediments revealed high densities of cells attached to the sediment particles in the black layer of site *JL* (Fig. 3c). The colonization of sediment from site *SN* and the brownish layer of *JL* was scarce (Fig. 3a,b). This corroborated the direct counts obtained with the Sybr Green I method.

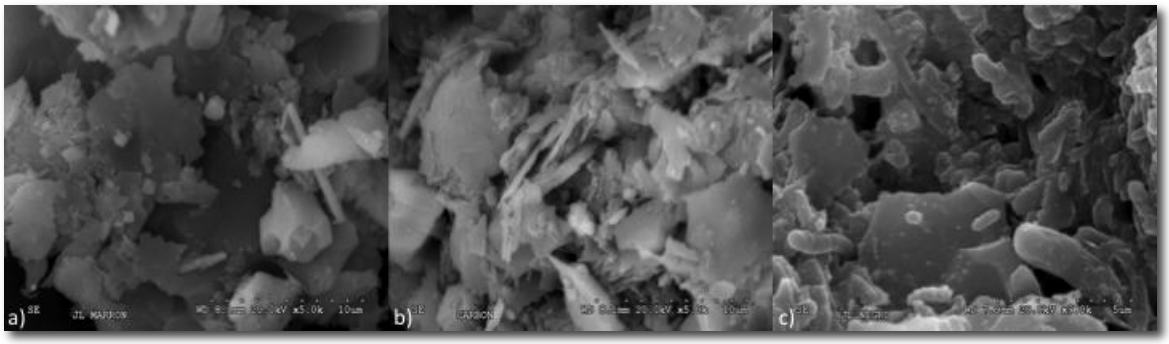


Fig. 3: Scanning electron micrograph (SEM) images: SN (a), JL brown (b) and JL black (c).

CARD-FISH

The sites investigated here had already been characterized in an earlier study by comparative 16S rRNA gene analysis, applying both fingerprinting by DGGE and comparative sequence analysis (42). FISH-based quantifications of particular taxa had first been hampered by high background due to the auto-fluorescence of sediment particles. These problems were solved in this study by applying the improved whole-cell hybridization technique CARD-FISH enabling the quantification of higher and lower taxa in a nested approach.

i) Domain-specific probes

First, oligonucleotide probes for the domains *Bacteria*, *Archaea* and *Eukarya* were applied to the sediment samples. In all cases *Bacteria* (>98%) dominated over *Archaea* (<2%) and *Eukarya* (below the detection limit) (Fig. 2b,f). As for the Sybr Green I total cell counts, great differences were found between the two sampling sites. In the *JL* sediment the average bacterial counts with probes EUB338 I-III (Fig. 2b) was $5.4x10^8$ cells g^{-1} wet weight of sediment, ranging from $1.61x10^9$ at 15 cm to $2.12x10^7$ at 45 cm, whereas in *SN* dam (Fig. 2f) the average was $8.9x10^6$, ranging from $1.6x10^7$ at 15 cm to $2.2x10^6$ at 45 cm.

ii) In-depth analysis of the bacterial community

An earlier study of Tinto sediments (42) had retrieved sequences belonging to five phyla of the domain *Bacteria*: *Proteobacteria* of the classes *Alpha-, Beta-, Gamma- and Deltaproteobacteria*, *Acidobacteria*, *Nitrospira*, *Actinobacteria* and *Firmicutes*. The selection of the phyla- and class-specific probes used in this study was done according to these previous findings.

Depth profiles of the respective bacterial groups are shown in Figure 2 (c,g). In *JL* dam sediments, *Deltaproteobacteria* was the most abundant group along the sediment core as detected by probe DEL495a-c (average $6.6x10^7$ cells g^{-1} wet weight). This class of *Proteobacteria* showed a strong variation with depth with two population maxima. The first peak ($3.3x10^8$ cells g^{-1} wet weight) was detected within the uppermost 5 cm and, after a sharp decline, a second population maximum ($1.1x10^8$ cells g^{-1} wet weight) was found in the most reduced layer (15-20 cm). The second most abundant group was *Firmicutes* detected with a probe mix consisting of LGC354a, LGC354b, CLIT135, CLOSTI and DSP648, with an average along the profile of $4.3x10^7$ cells g^{-1} wet weight. *Alphaproteobacteria* as detected by probe ALF968 averaged $4.2x10^7$ cells g^{-1} wet weight, *Gammaproteobacteria* as detected by probe GAM42a averaged $4.0\ x10^7$ cells g^{-1} wet weight and *Betaproteobacteria* as detected with probe BET42a averaged $2.6x10^7$ cells g^{-1} wet weight. In contrast, *Acidobacteria* (SS_HOL1400 probe) and *Actinomicetales* (HGC69a probe) appeared only in low numbers, $5.9x10^5$ and $7.7x10^6$ cells g^{-1}

wet weight respectively. Signals for probe NTR712 (*Nitrospira* group) were below the detection limit.

The depth profiles of *SN* dam sediments did not show the variation found in *JL*. In this core, numbers decrease homogeneously with depth. *Gammaproteobacteria* dominated (average $1.7x10^6$ cells g^{-1} wet weight) followed by *Alphaproteobacteria* with an average along the profile of $1.1x10^6$ cells g^{-1} wet weight. The rest of the groups were close to the detection limit, around 10^5 cells g^{-1} wet weight, in decreasing order: *Actinomycetales*, *Deltaproteobacteria*, *Firmicutes*, *Betaproteobacteria* and *Acidobacteria.*

iii) Genus-specificprobes

All genus-specific probes applied in this study were targeting genera for which sequences had been frequently retrieved in an earlier study (42). This included the deltaproteobacterial genera *Syntrophobacter* (probe Synbac824) and *Desulfurella* (probe DSU655), the genus *Desulfosporosinus* (probe DSP648) within the phylum *Firmicutes,* the alphaproteobacterial genus *Acidiphilium* (probe ACD638) and the gammaproteobactrial genus *Acidithiobacillus* (Thio820).

The genus-specific probing confirmed the great differences between the two sampling sites (Fig.2 d,h). In JL, the most abundant genus detected was *Syntrophobacter*. Cells were rod-shaped and had a size of about 2 by 1 µm. The average along the profile was $4.7x10^7$ cells g^{-1} wet weight, but just in the first layer the signal counts were $3.6x10^8$, representing 36% of the total *Bacteria* counts. *Syntrophobacter* spp. are propionate-degrading syntrophic bacteria that use sulfate as electron acceptor. They typically occur in environments with neutral pH. The other abundant genera were *Desulfurella* ($3.1x10^7$ cells g^{-1} wet weight, mostly rods of a size of 2 by 1 µm), and *Desulfosporosinus* ($2.8x10^7$ cells g^{-1} wet weight, curved rods of a size of 2-4 by 0.7 µm). The latter genus is known to encompass spore-forming sulfate reducing bacteria. Both genera showed higher numbers at those layers with higher pH and reducing redox potential (5-10 and 15-20 cm) (Fig.2 a,d). At *SN*, *Acidithiobacillus* spp. dominated with an average of $1.5x10^6$ cells g^{-1} wet weight along the profile. The hybridized cells were coccoid rods with a size of 1 by 0.7 µm. *Acidithiobacillus* spp. derive energy from the oxidation of reduced sulfur compound and/or ferrous iron, or the reduction of ferric iron under anoxic conditions. Finally, *Acidiphilium* spp. were also present with average numbers of $4.2x10^5$ cells g^{-1} wet weight (rods of a size of 2-3 by 1 µm).

DISCUSSION

We used a nested-probe approach for the quantification of the microbial community composition in sediments of Tinto River. The generally low abundance of *Archaea* agreed with similar results found in the sediments of mine tailing dumps (23). In a previous study of Río Tinto sediments (42), the archaeal diversity had been restricted to two genera: *Ferroplasma* spp. and *Thermoplasma* spp. Although Sanz *et al.* (43) had described the presence of methanogens in the sediments of the Tinto basin, our data suggest that *Archaea* play a minor role in the ecology of these sediments. Both *Ferroplasma* and *Thermoplasma* are usually found in bioleaching operations at lower pH (around 2) and at much higher iron and sulfate concentrations than those prevailing in Río Tinto sediments (13). Although algae, ciliates, flagellates, amoebae, and fungi have been reported in the water column and the biofilms of

the Río Tinto (1, 5), no *Eukarya* probe signal was detected by CARD-FISH, therefore, *Bacteria* seem to fully dominate the anoxic sediment layers of Tinto River.

When genus-specific probes were applied, a pronounced variability was detected between the two sampling sites. In *SN* sediments, where parameters such as pH and redox potential were similar to water column values, *Bacteria* related to the iron cycle prevailed, likely reflecting the higher availability of iron at low pH and oxidative redox potential. This included the genera *Acidiphilium* (ACD638 probe) and *Acidithiobacillus* (Thio820 probe), which both are capable of iron-reduction under anoxic conditions. No members of *Leptospirillum* genus, *Nitrospira*-like bacteria (Ntr712 probe), were detected. The common iron oxidizers in ARD environments are members of the aerobic genus *Leptospirillum* and the facultative anaerobe bacterium *Acidithiobacillus ferrooxidans,* which can oxidize iron and sulfur in aerobic conditions and reduce iron in anoxia. Our results confirm the dominance of the versatile *Acidithiobacillus ferrooxidans* in the sediments over the strictly aerobic *Leptospirillum* spp. that usually dominates in extremely acidic oxic ARD environments (7, 13).

At *JL* dam, where the iron solubility is lower due to higher pH and reducing redox potential, microbes of the sulfur cycle were most abundant. Here, sulfate-reducing bacteria (SRB) of the genera *Desulfosporosinus* (DSP 648 probe) and *Syntrophobacter* (Syn824 probe) as well as sulfur reducers of the genus *Desulfurella* (DSU655 probe) predominated, showing the importance of sulfur cycling in these sediments. *Syntrophobacter*-affiliated 16S rRNA gene sequences (42) (HQ730668.1, HQ730666.1) have been retrieved in several acidic environments before (11, 17, 26), suggesting that not yet cultured relatives of *Syntrophobacter* might be well adapted to acidic conditions in contrast to the general neutral pH environments where they thrive.

The analyses of both brown and black layers in the JL sediments showed a significant reduction in the last ones for dissolved metals, iron and sulfate in the black layers, which run parallel to the SRB abundance. *Desulfurella* and *Desulfosporosinus* genera abundance peaked perfectly with those black layers where the pH values and acetate concentration increased and the redox potential, the sulfate and the dissolved metals decrease. Our results suggest that the attenuation of ARD characteristics in the reduced black layers is biologically driven by SRB (Fig.4).

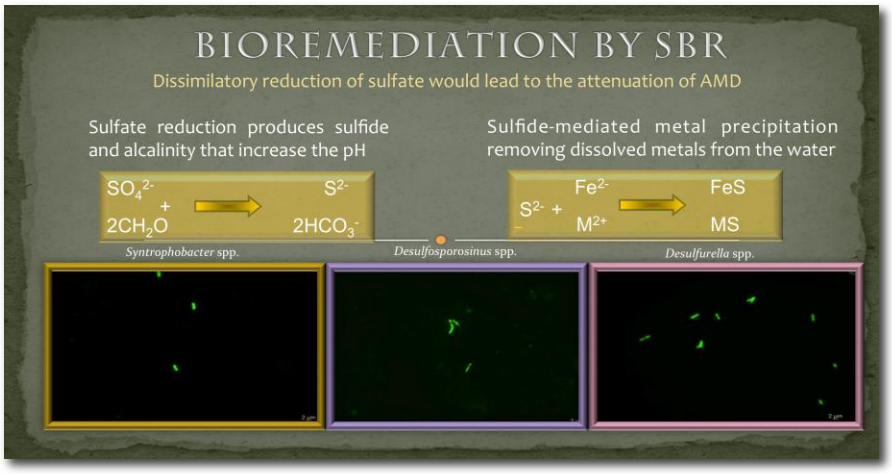


Fig. 4: ARD bioremediation by SBR model.

The dissimilative reduction of sulfate, driven by identified sulfate- and sulfur-reducing bacteria leads to a consumption of protons thus, increasing alkalinity, and to sulfide formation. In addition, there is a sulfide-mediated metal precipitation: the sulfide reacts with the dissolved iron and other heavy metals which can form black amorphous precipitates (FeS) and metallic sulfides (MS). These results agree with previous studies where remediation in AMD associated with SRB metabolism was observed (14, 16, 21, 39).

In summary, the CARD-FISH quantifications performed in this study underline the great differences between microbial communities of the two Río Tinto sediments studied that had been first indicated by DGGE and comparative sequence analysis. These differences correlate with their physico-chemical characteristics, which would correspond to different hydrology at both sites. In *JL* dam, the location, thickness and values of pH and redox potential of the oxidized and reduced zones vary temporally, but stratification has been routinely observed. In contrast, sediments in *SN* have been more homogeneous. Different interpretations can be put forward. On the one hand, the two sampling sites have different hydrology. Site *SN* is a stream located close to the source of the river with a constant water flow containing high concentration of heavy metals, mainly iron. Therefore, the sediments characteristics are close to those of the water column strongly buffered by ferric iron. In contrast, the site *JL* is located 5 km downstream, with an increasing flow due to the addition of neutral tributaries which lower the iron concentration, albeit with a strong seasonal variability. One of the tributaries upstream *JL* contains the waters from the municipal wastewater treatment plant of Nerva. This significantly increases the total organic content (TOC) of the waters. The combination of both factors could explain the differences detected in both types of sediments. At *JL* dam, with seasonal variability, higher TOC and lower iron concentration, microbial reduction processes such as sulfate reduction increase the pH. This effect would take place firstly in microniches and then expands to macroscopic conditions. That would explain the banded sediment at *JL* dam, which would correspond to the fluctuating conditions of the input water. Within them, SRB communities reduce the dissolved sulfates to sulfides that precipitate iron and heavy metals, performing a local natural bioremediation of Tinto River.

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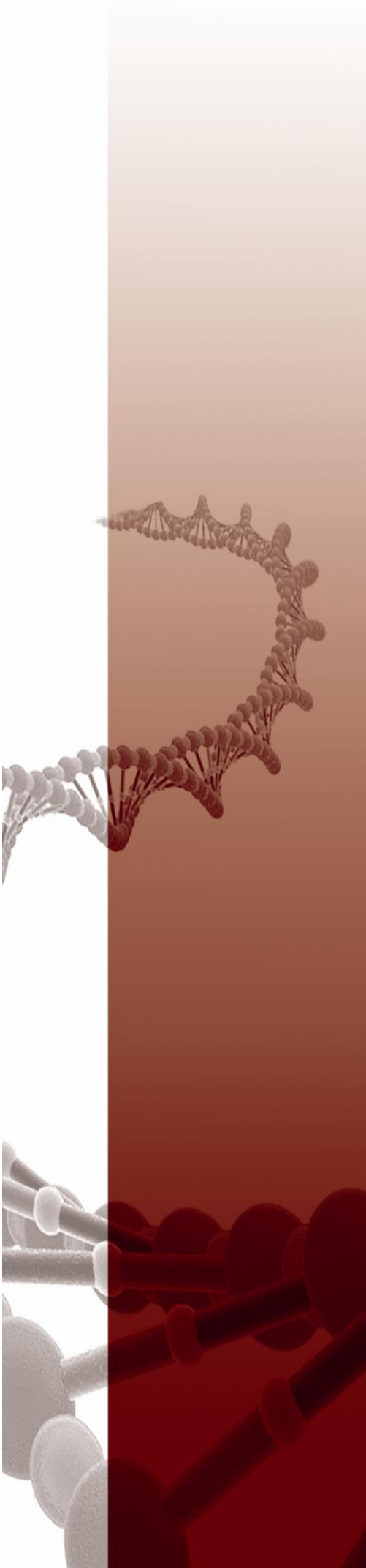
CHAPTER 3: Screening of Anaerobic Activities in Sediments of an Acidic Environment: Tinto River



INTRODUCTION

The exploration of extreme environments has led to the discovery of microbial activities in habitats that only a few years ago were considered uninhabitable. As a consequence, interest in their ecology has grown. The Tinto river is a natural acidic rock drainage (ARD) environment located in the Iberian Pyritic Belt (IPB), in south-western Spain. The bio-oxidation of metal-sulfides in this system results in waters of low pH (pH 2.3), high concentrations of sulfate (6 g L^{-1}), ferric iron (2.3 g L^{-1}), and other heavy metals (Cu: 0.1 g L^{-1}; Zn: 0.23 g L^{-1}, etc.). Apart from the ecological interest of extremophiles; the Tinto River is also of interest in the areas of biogeochemistry (exploring the nature of the microbial communities associated with ARD), biotechnology (bioleaching processes), microbiology (acidic environments offer an almost unique opportunity to reveal habitat biological complexity) and Mars-analogue studies (common mineralogy).

Recent geomicrobiological studies of the Tinto river have shown a high level of microbial diversity in the water column, both in prokaryotic and eukaryotic organisms. Eighty percent of the prokaryotic biomass in the water column is composed of three genera of bacteria, *Leptospirillum*, *Acidithiobacillus* and *Acidiphilium* (20), all of which are involved in the iron cycle. Macroscopic brownish-filamentous structures, streamers, are present along the river, composed mostly of those three bacterial genera enmeshed in a matrix of exopolysaccharides and mineral particles (19). A wide variety of eukaryotes, which include algae, ciliates, flagellates, amoebae, fungi and rotifers have been reported. Eukaryotic algae, which contribute to over 60% of the river biomass (32), are mainly chlorophytes but also filamentous algae forming green-phototrophic filaments (1-3). Heterotrophic protists -mixotrophic flagellates, ciliates or amoebas- are also widely distributed along the river. Among decomposers, fungi are the most abundant and both unicellular and filamentous forms are present (31, 32). The only animal found in the river is a species of bdelloid rotifer related to the genus *Rotifera* (60).

Most of the interest in these ecosystems has focused on the aerobic iron- and sulfur-oxidizing microorganisms due to their bio-mining applications. Despite their ecological interest, the characterization of the anoxic sediments from acidic environments, such as those of the Tinto river, had been neglected, with few exceptions (9, 22). Our group has recently started an in depth Tinto River sediments study to understand how the element cycles work under anaerobic conditions and to find potential biotechnological applications for the microorganisms thriving in them. First, an extensive survey of the Tinto River sediment microbiota based on comparative sequence analyses of 16S rRNA genes was performed at two contrasting sampling sites (*SN* and *JL* dams) using two culture independent approaches: DGGE and cloning of 16S rRNA genes (46). Microorganisms involved in the iron (*At. ferrooxidans*, *Sulfobacillus* spp., *Ferroplasma* spp., etc.), sulfur (*Desulfurella* spp., *Desulfosporosinus* spp., *Thermodesulfobium* spp., etc.) and carbon (*Acidiphilium* spp., *Bacillus* spp., *Clostridium* spp., *Acidobacterium* spp., etc.) cycles were identified and their distribution correlated with physicochemical parameters of the sediments. Hybridization with domain-specific probes showed that *Bacteria* (>98%) dominated over *Archaea* (<2%) (45). Specific probes showed large differences between dams. At *SN* where the pH and redox potential are similar to that of the water column (pH 2.5 and +300 mV), the most abundant organisms were identified as iron-reducing microorganisms: *Acidithiobacillus* spp. and *Acidiphilium* spp., given the higher

solubility of iron at low pH. At the *JL* dam, characterized by a higher pH (4.2 - 6.2), more reducing redox potential (50, -210 mV) and a lower solubility of iron, members of sulfate-reducing genera *Syntrophobacter, Desulfurella* and *Desulfosporosinus* dominated.

With these previous studies based on molecular ecology methods, the prokaryotes thriving in the sediments have been identified, quantified and their corresponding metabolism inferred. A model of the iron, sulfur and organic matter cycles operating in the sediments was proposed, suggesting the presence of iron-reducers, methanogens, nitrate-reducers, hydrogen producers and sulfate-reducing bacteria (SRB). However a culture-dependent approach is needed to prove the existence microbes performing those potential anaerobic activities and identify them. Iron reducers are common at low pH and have been well studied in ARD-sediments (9), but the other detected activities, unusual at low pH, have received less attention. Therefore, the objectives of this study were, firstly, to investigate the viability of these activities in ARD-related sediments throughout the river, then, to identify the responsible organisms and finally, if possible, to isolate those with potential biotechnological applications.

MATERIALS AND METHODS

Sampling sites

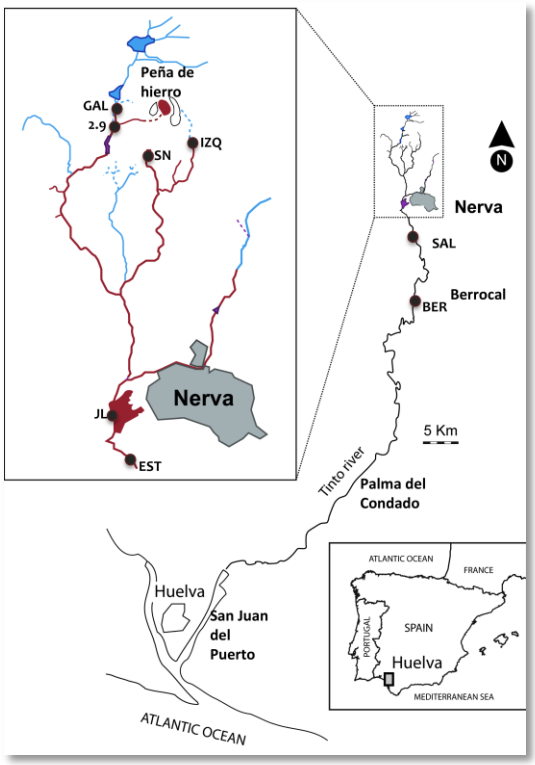


Fig. 1: Map of Tinto river sampling sites of this study.

Samples were collected from eight sampling sites, *JL* (37.691207N, 6.560587W), *SN* (37.72173N, 6.557465W), *GAL* (37.725362N, 6.561853W), *BER* (37.601346 N, 6.549933W), *SAL* (37.669622N, 6.550212W), 2.9 (37.7242N, 6.56207W), *EST* (37.68832N, 6.563773W), and *IZQ* (37.72891N, 6.550255W) along the Tinto River (Fig.1) in June 2009 and May 2010. The bed of Tinto River is rocky, therefore samples were taken where there was sufficient sediment accumulation (10

cm). The redox potential (E) and pH of the drill core samples were measured *in situ* with E and pH probes, connected to a Thermo Orion 290A potentiometer placed into the fresh sediment just after extraction. In general all sampling sites presented a pH range between 2 and 3 and redox between 200 and 300 mV with the exception of *JL* at which pH was 5 and redox potential between -50 and 80 mV.

Enrichment cultures

Enrichments were prepared anaerobically using an anaerobic chamber. Approximately 4 g of sediment were added to 50 mL glass serum bottles with 20 mL of media and capped with butyl rubber stoppers. The headspace was then degassed with a N_2/CO_2 (80:20) mixture. Enrichments were incubated at 30°C in the dark. The growth media used were selective for various microbial activities. For methanogenesis, the basal medium previously described (47) was used at pH 6. To stimulate specific methanogenic groups, six different carbon sources were used: a) H_2+CO_2 (80:20); b) volatile fatty acids (VFAs) (0.5 g L^{-1} of each sodium salts of acetate, propionate and butyrate); c) mixture (M) (g L^{-1}) (0.7 sodium formate, 0.5 sodium acetate, 0.3 sodium propionate, 0.3 sodium butyrate, 0.5 methanol, 2 sucrose and 0.7 sodium lactate); d) 2 g L^{-1} sodium acetate; e) 1.5 g L^{-1} sodium formate; and f) 1 g L^{-1} methanol. For sulfate reduction, the medium contained (g L^{-1}): 2 sodium lactate, 2 $KHCO_3$, 2 yeast extract, 2 $MgSO_4 \cdot 7H_2O$, 1.5 Na_2SO_4, 0.5 K_2HPO_4, 0.1 $CaCl_2$, 0.48 $(NH4)_2Fe(SO_4)_2 \cdot 6H_2O$, and 1 mL of micronutrients solution (47). Enrichments for sulfate reducing bacteria were conducted at pH 5 and 7. Enrichments for both autotroph and heterotroph denitrification were conducted. Both the heterotrophic (3635) and the autotrophic (4544) denitrifying media were prepared as described previously. In both media, final pH 5 and 7 were assayed. The medium used for hydrogen production was (g L^{-1}): 0.3 NH_4Cl, 0.3 K_2HPO_4, 0.1 $MgSO_4$, 0.5 $NaHCO_3$, 0.2 sucrose, 0.1 meat extract, 0.5 yeast extract, and 1 mL of micronutrients solution (47). Cysteine was used as reducing agent (0.5%) in all media.

Analytical Measurement

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

Isolation

Hydrogen-producers and sulfate-reducers, both of biotechnological interest, were isolated from enrichments. When activity was detected, sequential transfers were made into fresh medium. Those producing significant amount of H_2 or those with FeS precipitates were isolated by inoculation on agar plates and incubation at 30°C in anaerobic jars. The solid media were the same as those mentioned above plus 1.5% bacteriological agar. Single colonies were picked and inoculated into 20 mL serum bottles with the selective media.

DNA extraction/16S rRNA gene amplification

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

Denaturing gradient gel electrophoresis (DGGE)

The V3 to V5 variable regions of the 16S rRNA gene were amplified with the primers set 341F (GC)-907R (annealing temperature (T_a=52°C) for *Bacteria* and 622F (GC)-1100R (T_a=42°C) for *Archaea*. Primers 341F (GC) and 622F (GC) included a GC clamp (3938). The amplification reaction was performed according to the Taq DNA Polymerase protocol (Promega, USA). The PCR conditions were as follows: 5 min of initial denaturation at 94°C; 30 cycles at 94°C for 1 min, 52 or 42°C for 1 min and 72°C for 2 min; followed by 10 min final incubation at 72°C. DGGE analysis was carried out using a D-Code Universal Detection System instrument (Bio-Rad) according to the manufacturer´s instructions (Bio-Rad). Polyacrylamide (6%; 37.5:1 acrylamide-bisacrylamide) gels with a 30 to 60% urea-formamide denaturant gradient (100% urea-formamide contains 7 M urea and 40% deionized formamide) were used in 1x TAE (Tris-Acetate-EDTA) buffer, pH 7.4, at 200 V for 4 h at 60°C. Gels were stained with ethidium bromide and visualized under UV illumination. The bands were cut from the gel with a sterile blade and placed in vials with 100 µL of MilliQ water. DNA was allowed to diffuse into the water at 4°C overnight. Five microliters of the eluate were used as a DNA template in a 50 µL PCR with the primers 341F/907R. The PCR products were purified with Jetquick PCR Product Purification Spin Kit (Genomed, Germany), according to the manufacturer´s instructions.

Phylogenetic analysis

Samples were sequenced using a Big-Dye sequencing kit (Applied Biosystems). Sequences were checked with the program Finch TV (Geospiza, USA). Complete 16S rRNA sequences were assembled using DNAbaser program. Comparative analysis was done using BLAST from NCBI (http://www.ncbi.nlm.nih.gov/Blast) and Sequence Match tool from Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/).

Nucleotide sequence accession numbers:

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

RESULTS AND DISCUSSION

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

Methanogenesis

A screening of 48 enrichments was performed, analyzing 8 sampling sites and 6 different carbon sources. The results are summarized in the Table 1.

Table 1: Summary of anaerobic activities and sampling sites.

Sites	Methanogenesis	Sulfate-reduction	Denitrification	Hydrogen-production
JL	-	+	+	+
SN	-	+	+	+
GAL	+	+	+	+
BER	+	+	+	+
SAL	+	+	ND	ND
2.9	+	-	ND	ND
EST	-	-	ND	ND
IZQ	+	-	ND	ND

[+]presence in any of the conditions tested [-]absence in all the conditions assayed [ND] not determined

Eleven enrichments (Fig. 2a) showed methane production (nmol CH_4 per gram wet sediment and day): *SAL* with mixture M (32.9), *2.9* with methanol (24.5), *GAL* with VFA (2.8) and mixture M (29.4), *BER* with H_2+CO_2 (2.5), VFA (1.2), sodium acetate (43.1), sodium formate (25.4) and methanol (30.8), and *IZQ* with sodium formate (37.4) and methanol (5.3). The positive enrichments were transferred once, total DNA was extracted and DGGE was run (Fig. 3). Twenty-two bands were excised from the gel; twelve were successfully amplified and sequenced (Table 2). *SAL* (M) and *2.9* (methanol) samples did not yield amplifiable bands.

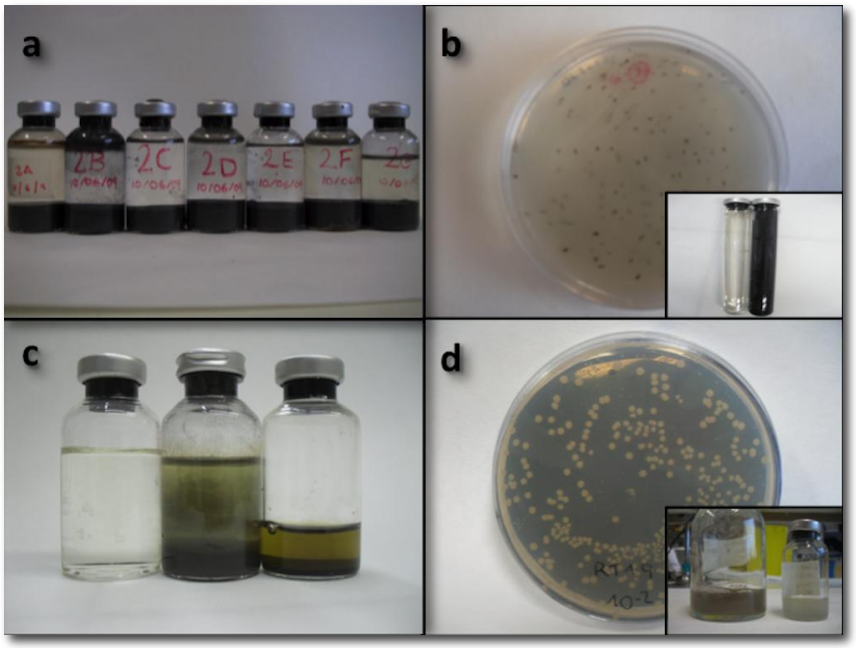


Fig. 2: Images of enrichment cultures of the tested activities: a methanogenesis, b sulfate-reduction, c nitrate reduction, d hydrogen-production. Sulfate-reduction can be detected by the black precipitates and nitrate-reduction by the green coloration.

Table 2: Phylogenetic affiliation of archaeal DGGE sequences from methanogen enrichments.

DGGE band	Accession number	Isolation Medium	Phylogenetic assignment Class, Family, Genus species; % similarity
BER(1-3)_CF	JQ271588 JQ271589 JQ271590	Methane/Methanol	*Thermoprotei,Thermofiliaceae , Uncultured archaeon* ArcIV_cloneF06 < 95%
BER(1,2,4)_CD	JQ271591 JQ271592 JQ271594	Methane/Acetate	*Methanomicrobia, Metanosarcinaceae, Methanosarcina barkerii* strain Sar; 99-100%
BER3_CD	JQ271593	Methane/Acetate	*Methanomicrobia, Metanosarcinaceae, Uncultured methanosarcina* sp. clone Soil113B_10 ; 94%; *Methanosarcina barkerii* strain Sar; 94%
IZQ1_CE	JQ271595	Methane/Formate	*Methanobacteria, Methanobacteriaceae, Methanobrevibacter arboriphilus* strain AZ; 100%
IZQ2_CE	JQ271596	Methane/Formate	*Methanobacteria, Methanobacteriaceae, Methanobrevibacter arboriphilus* strain AZ ; 96%
GAL(1,2)_CB	JQ271597 JQ271598	Methane/VGA	*Thermoprotei,Thermofiliaceae, Uncultured archaeon* clone ArcIV_cloneF06 ; 99%

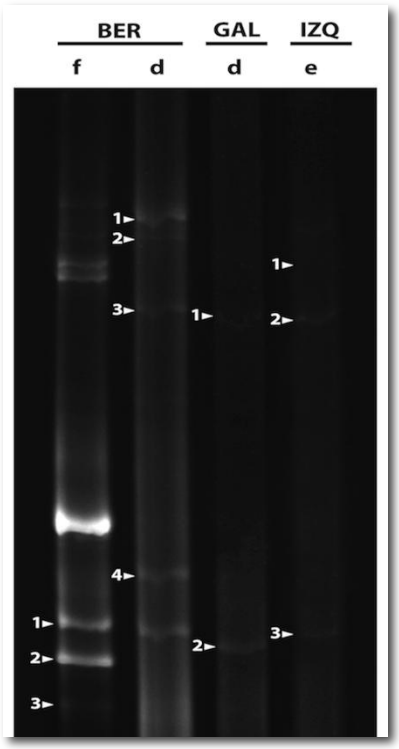


Fig. 3: DGGE fingerprints of 16S rRNA obtained with domain-specific primers for Archaea from methanogenic enrichments. Sampling locations are displayed on the top of the lanes followed by the electron donor nomenclature used in the sequences: methanol (f), acetate (d) and formate (e). Letters within lances correspond to the sequence nomenclature shown in Table 2.

Sequences were affiliated to both archaeon phyla described: *Crenarchaeota* and *Euryarchaeota*. Regarding the *Crenarchaeota,* some sequences retrieved from *GAL* and *BER* sites were affiliated to the family *Thermofiliaceae*, which comprise known neutrophilic and hyperthermophilic archaea (11). Despite these features, uncultured relatives of our *Thermofiliaceae*-related sequences have been detected in acidic sediments (FN870267) (33). Related sequences have also appeared linked to methanogenic habitats (AJ576215) (25), (AB243802) (43) and (EU155990) (12).

It was possible to identify phylogenetically down to the genus level some of the *Euryarchaeota* sequences. The nearest cultivated relatives to the DGGE bands at the sampling site *IZQ* incubated with formate was *Methanobrevibacter arboriphilus*, being the formate a common *M. arboriphilus* substrate (5, 42). This is to our knowledge the first report of this neutrophilic methanogen in an acidic environment. On the other hand, the nearest cultivated relatives to sequences of sampling site *BER* incubated with acetate was *Methanosarcina barkerii*. The presence of *Methanosarcina* and the absence of *Methanosaeta*, the other methanogenic acetoclastic genera, can be attributed to: (i) the high acetate concentration used in the enrichment medium, which is much higher than the Ks of *Methanosarcina* for acetate, which outcompete *Methanosaeta* (Ks and μmax $5x10^{-3}$ mol $L^{-1}$and $2.5x10^{-2}$ h^{-1} for *Methanosarcina* versus $0.5x10^{-3}$ mol L^{-1} and $3x10^{-3}$ h^{-1} for *Methanosaeta*) (ii) the ability of *Methanosarcina* to tolerate low pH conditions (34), consistent with previous studies (18, 23, 52). Remarkably, *Ms. barkeri* was identified in a methanol-spiked microcosm inoculated with Tinto River sediments (48).

Methanogenesis is usually believed to be inhibited by low pH, however, several studies have

shown methanogenesis taking place at acidic conditions, even at pH as low as 4.5 (10, 16, 18, 21, 29, 59). In fact, while the Gibbs energy of the hydrogenoclastic methanogenesis remains constant at different pH, the acetoclastic methanogenesis is energetically more favourable at pH below 4.5 (15), which indicates that once the methanogens are able to support a low pH environment, the process is thermodinamically possible and favourable.

Sulfate-reduction

A screening was performed with samples from 8 sites incubated at pH 5 and 7 with lactate as an electron donor. In this set of 16 bottles, six showed activity (Table 1) at pH 7, as shown by the occurrence of FeS black precipitates (Fig. 2b). Activity was also detected for enrichments at pH 5 from 3 different sites.

The enrichments were inoculated on agar plates (SRB media at pH 7 and 5). Positive results were only obtained in samples plated on agar at pH 7. Black colonies (Fig. 2b) were picked and inoculated in specific medium. Isolates were analyzed by 16S rRNA amplification and sequenced to determine their identity. Sequences were affiliated within the genera *Clostridium* and *Desulfotomaculum* (phylum *Firmicutes*, order *Clostridia*) (Table 3).

Table 3: Phylogenetic affiliation of sequences from sulfate-reducer isolates.

Isolate	Accession number	Isolation Medium	Phylogenetic assignment Class, Family, Genus species; % similarity
JL(1,2,7)_S	*JQ271537-38-43*	SO_4^{2-}/Fe^{2+}	*Clostridia,Clostridiaceae, Clostridium* sp. OkiF101; 98%
JL3_S	JQ251539	SO_4^{2-}/Fe^{2+}	*Clostridia, Clostridiaceae, Clostridium bifermentans* strain *DPH-1*; 99%
JL4_S	JQ271541	SO_4^{2-}/Fe^{2+}	*Betaproteobacteria, Alcaligenaceae*; < 90%
JL5_S	JQ271542	SO_4^{2-}/Fe^{2+}	*Clostridia, Clostridiaceae, Clostridium bifermentans* strain *DPH-1*; 93%
SAL(1-3)_S	*JQ271544 JQ271546*	SO_4^{2-}/Fe^{2+}	*Clostridia, Peptococcacea, Desulfotomaculum sp.* Iso-W2; 99%; *Clostridium celerescens* clone IrT-JG1-12; 99%
GAL(1,2,3,5)_S	JQ271547-48-50-52	SO_4^{2-}/Fe^{2+}	*Clostridia, Clostridiaceae, Clostridium bifermentans* strain DPH-1; 99-100%
GAL4_S	JQ271551	SO_4^{2-}/Fe^{2+}	*Clostridia, Peptococcaceae, Desulfotomaculum sp.* Iso-W2; 99%; *Clostridia,Clostridiaceae, Clostridium celerescens* clone IrT-JG1-12; 99%

Desulfotomaculum is a dissimilatory SRB able to form terminal and subterminal spores (13). In permanently anoxic habitats the non-sporeformers are dominant, but when the habitats are variable the sporeforming sulfate reducers dominate (58), *Desulfotomaculum* is able to survive dryness and oxic conditions for many months, even years. Interestingly enough, the closest relatives to our sequences are found in metalliferous organic soils (DQ479411), coal mines (HQ827821) and polluted estuaries (DQ677019) (30). The sequences had a high similarity with *Desulfotomaculum guttoideum* and *Desulfosporosinus orientis*, a SRB (51) previously found at Tinto River (19, 46).

Previous studies in the extreme environment of Tinto River had suggested the presence and importance of a sulfate-reducing microbial community in the anaerobic zones of the Tinto River (46). This is interesting from a biotechnological point of view, as such communities can be used in the bioremediation of acidic waters with a high heavy metals content (7, 14, 26) and from an ecological point of view, due to the fact that SRB were supposed to prefer a neutral

environment. In the last decades, considerable evidence has revealed that sulfate-reduction at pH values below 5 is possible (28) and our results confirm the effective adaptation of SRB at low pHs by showing that active acidotolerant sulfate-reducers thrive in Tinto River sediments.

Denitrification

Biological denitrification, both assimilative and dissimilative, is the biological process for removing nitrate. Typically in most environments, nitrate is used as an electron acceptor by facultative heterotrophic bacteria comprising many diverse genera (*Pseudomonas*, *Alcaligenes*, *Bacillus*, etc.) under anoxic conditions, where organic matter provides electron donors to reduce nitrate to dinitrogen gas (37). In contrast to the great diversity of heterotrophic denitrifiers, there are just a few autotrophic denitrifiers, able to use hydrogen, ferrous iron, or reduced sulfur compounds as electron donors.

As previously mentioned, autotrophic and heterotopic denitrification at pH 5 and 7 were assayed at selected sampling sites (*SN, JL, BER* and *GAL*). In this set of 16 enrichments, denitrification activity was followed by nitrate (NO_3^-), reduction and nitrite (NO_2^-) and nitrous oxide (N_2O) production. Eleven of the enrichments showed activity with the exception of all *GAL* enrichments and *SN* enrichments at pH 5 in autotrophic medium. Positive activity correlated with the appearance of green colour in the bottles (Fig. 2c). An increment in the pH was detected in the enrichments with an average of 0.02 units/day. Nitrate was consumed during the assay with an average of 1 mmol per mg bacterial wet weight and day. Nitrite was detected, but in a 7-fold lower concentration in the autotrophic samples in comparison with the heterotrophic ones. Nitrous oxide accumulated at 0.01 mmol per mg bacterial wet weight and day. A larger accumulation of nitrous oxide, 5-fold, was detected in the enrichments growing at pH 5 versus those growing at pH 7.

The optimum pH range for complete reduction of nitrate to nitrogen gas (N_2) is considered to be between 6 and 8. In acid soils, below this optimal pH range, it has been reported that final steps of the denitrification are inhibited. In these environments, the proportion of intermediate products such as nitrite (8, 40) and nitrous oxide (50) increase with respect to dinitrogen gas. A study with enrichment cultures suggested that denitrification occured in ARD streams, and might reduce acidity (6), as confirmed in this assay.

Microbial populations were analyzed by DGGE to study the diversity and taxonomic position of the responsible microbes. From the eleven positive enrichments, just eight yielded amplifiable DNA (Fig. 4).

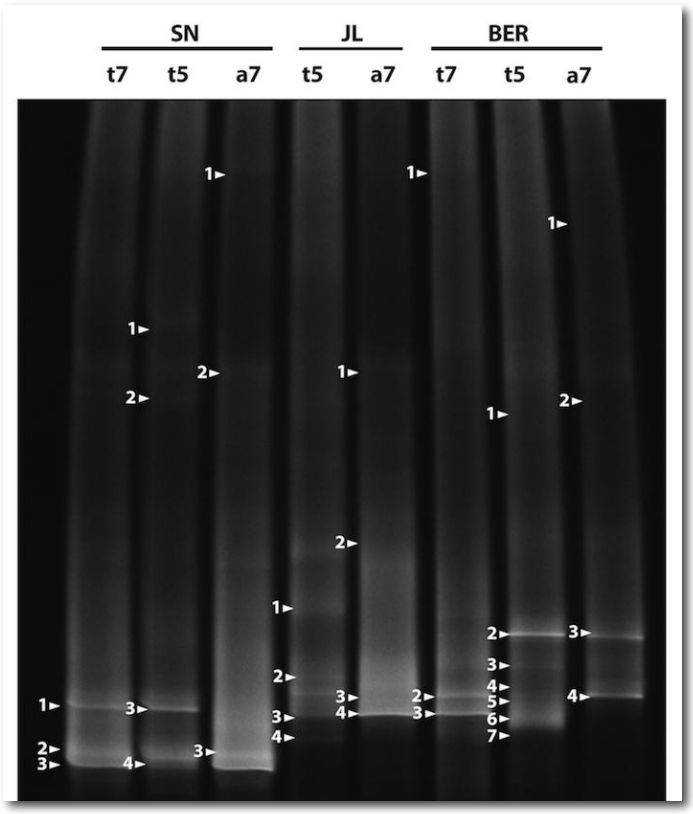


Fig. 4: DGGE fingerprints of 16S rRNA obtained with domain-specific primers for Bacteria from denitrifying enrichments. Sampling locations are displayed on the top of the lanes followed by medium nomenclature: t (heterotrophic medium), a (autotrophic), 5 (pH 5) and 7 (pH 7) Letters within lances correspond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

Forty-nine bands were excised from the gel; thirty-two were successfully amplified and sequenced. As shown in Table 4, most of the sequences were affiliated with the phylum *Firmicutes*, with just one exception related to the *Betaproteobacteria* class. The organisms belonged to the genera *Bacillus*, *Paenibacillus*, *Lysinobacillus*, *Ammoniphilus*, *Rummelibacillus* (*Bacilli* class), *Clostridium*, *Desulfitobacterium, Sedimentibacter* (*Clostridia* class), and *Alcaligenes* (*Betaproteobacteria* class), previously reported as denitrifying bacteria (55, 56). The closest relatives to some of the sequences, such as *Bacillus* spp. (JN082261), *Sedimentibacter* spp. (EF464626) and *Desulfitobacterium* spp. (JF346160), were found in ARD systems. The most diverse communities appeared in the heterotrophic enrichments at pH 5, which had nine different bacterial species. In the rest of the enrichments (autotrophic media at both pHs and heterotrophic one at pH 7), just *Bacillus* and *Paenibacillus* appeared.

Table 4: Phylogenetic affiliation of bacterial DGGE sequences from denitrifying enrichments

Band	Accession number	Isolation Medium	Phylogenetic assignment Class, Family, Genus species; % similarity
JL1_Na5	JQ271565	$NO_3^-/S_2O_3^{2-}$	*Bacilli, Paenibacillaceae, Paenibacillus sp.* enrichment culture clone 9; 99%
JL(1-4)_Na7	JQ271566 JQ271569	$NO_3^-/S_2O_3^{2-}$	*Bacilli, Bacillaceae, Bacillus sp.* cp64; 100%
JL1_Nt5	JQ271570	NO_3^-/Y.E.	*Clostridia, Clostridiaceae, Sedimentibacter sp.* enrichment culture clone B4120; 96%
JL2_Nt5	JQ271571	NO_3^-/Y.E.	*Bacilli, Bacillaceae, Lysinobacillus fusiformis* strain B116; 98%
JL3_Nt5	JQ271572	NO_3^-/Y.E.	*Bacilli, Paenibacillaceae, Ammoniphilus sp.* CC-RT-E; 98%
JL1_Nt7	JQ271573	NO_3^-/Y.E.	*Bacilli, Bacillaceae, Bacillus sp.* HZ-B; 99%
SN(1-3)_Na7	JQ271574-76	$NO_3^-/S_2O_3^{2-}$	*Bacilli, Bacillaceae, Bacillus sp.* HZ-B; 99%
SN1_Nt5	JQ271577	NO_3^-/Y.E.	*Betaproteocbacteria, Comamonadaceae , Delftia tsuruhatensis* strain WYLW2-1; 91% ; *Betaproteocbacteria, Alcaligenaceae,* Alcaligenes *sp;* 91%
SN1_Nt7	JQ271578	NO_3^-/Y.E.	*Bacilli,* Bacillaceae, *Bacillus sp. HZ-B;* 99%
BER(1-4)_Na7	JQ271553 JQ271556	$NO_3^-/S_2O_3^{2-}$	*Clostridia, Clostridiaceae, Clostridium jejuense* strain *HY-35-12;* 96%
BER1_Nt5	JQ271557	NO_3^-/Y.E.	*Clostridia, Clostridiaceae, Clostridium jejuense strain HY-35-12;* 97%
BER(2,3,4)_Nt5	JQ271558 JQ271560, JQ271561	NO_3^-/Y.E.	*Clostridia, Peptococaceae, Desulfitobacterium sp. enrichment culture clone* CEB3; 97%
BER(1-3)_Nt7	JQ271562 JQ271564	NO_3^-/Y.E.	*Bacilli, Paenibacillaceae, Paenibacillus sp. enrichment culture clone S16;* 88%

In previous studies of the boreholes in the Tinto River, activity in the enrichments of thiosulfate-oxidizers using nitrate as an electron acceptor was detected, but the organisms responsible for these activities were neither isolated nor characterized (17). Our study is the first to confirm the presence, activity and diversity of a denitrifying community in Tinto River sediments.

Hydrogen-production

Because hydrogen is released as a consequence of different anaerobic metabolisms, four of the selected sampling sites (*JL*, *SN*, *BER* and *GAL*) were assayed for hydrogen production. Enrichments showed significant production of H_2 (300-400 mL g^{-1} Chemical Oxygen Demand consumed) in all the cases. Twelve colonies per sample were isolated from agar plates (Fig. 2d) and analyzed by 16S rRNA gene amplification (Table 5).

Table 5: Phylogenetic affiliation of sequences from hydrogen-producers isolates.

Isolates	Accession number	Isolation Medium	Phylogenetic assignment Class, Family, Genus species; % similarity
BER1_H	JQ271579	Sucrose	*Clostridia, Clostridiaceae, Clostridium* sp. enrichment culture clone NHT ; 99%
BER2_H	JQ271580	Sucrose	*Clostridia, Clostridiaceae, Clostridium* sp. enrichment culture clone NHT; 100%
BERGAL_H	JQ271581	Sucrose	*Clostridia, Clostridiaceae, Clostridium beijerinckii* strain RZF1108 99%
GAL1_H	JQ271582	Sucrose	*Clostridia, Clostridiaceae, Clostridium bifermentans* strain: JCM 1386.; 100%
GAL2_H	JQ271583	Sucrose	*Bacilli, Bacillates incertae sedis, Rummeliibacillus pycnus* strain NBRC 101231; 98%
SN1_H	JQ271584	Sucrose	*Clostridia,Clostridiaceae, Clostridium celerecrescens clone IrT-JG1-12; 99%; Clostridia, Peptococcaceae, Desulfotomaculum* sp. Iso-W2; 99%
SN2_H	JQ271587	Sucrose	*Clostridia,Clostridiaceae,Clostridium sp.* FGH; 99%
JL1_H	JQ271585	Sucrose	*Clostridia, Clostridiaceae,Clostridium beijerinckii strain:* HU-2; 99%
JL2_H	JQ271586	Sucrose	*Clostridia, Clostridiaceae, Clostridium beijerinckii strain:* HU-2 ; 100%

The 48 sequences, grouped into 9 OTUs, clustered in the *Firmicutes* phylum. Except one isolate belonging to the *Rummenibacillus* genus (*Bacilli/ Bacillales incertae sedis*), the rest of the isolates were affiliated to the *Clostridium* genus (*Clostridia/Clostridiaceae*).

Hydrogen can be produced by anaerobic bacteria, including obligate, facultative and photosynthetic strains (41). This is often done through dark- or photo-fermentation. Dark fermentation, also called acid fermentation is the conversion of organic substrates to H_2 by anaerobic microorganisms, such as *Clostridium*, *Syntrophophobacter* or *Enterobacter*. *Clostridium* species are the most well-known microorganisms involved in H_2 production by acid fermentation (24, 53, 57). They usually ferment sugars or proteins to acids (acetate, propionate, butyrate, lactate), alcohols (ethanol), or use carbon dioxide and molecular hydrogen.

In previous studies of the boreholes in the Tinto River (4), the fluid extracted from those underground habitats contained dissolved gases, such as H_2, N_2O, and CH_4. The chemical origin of H_2 was proposed to be derived from a water-rock interaction, but the identification of hydrogen-producing bacteria, such as *Syntrophobacter* spp. and *Clostridium* spp., in the previous molecular ecology study of Tinto River sediments (46) suggested the possibility of a biological production of this gas. This study confirms the bioproduction of this gas through culture-dependent techniques.

Environmental and biotechnological implications

Our study proves the existence of the anaerobic activities previously inferred by molecular biology methods (46) and identifies the responsible microorganisms. Additionally, it confirms

the bioproduction of H_2, CH_4 and N_2O, some of the gases detected in a borehole performed in the deposits rock matrix of Tinto River (4).

Together with the ecological implications of the results shown in this study, many potential biotechnological applications arise. Methanogenesis is an important process in wastewater treatment, and methanogens with a tolerance for acidic or metalliferous conditions could be very useful in remediating acidic metal polluted waste streams. Biological processes using renewable sources for hydrogen-production are more economical and environmental friendly than the physical-chemical processes presently used (36, 49), so interest in biological hydrogen-production is increasing. Additionally, SRB can be used for remediation of acidic water with high heavy metals content or radionuclides (26, 27, 39, 54).

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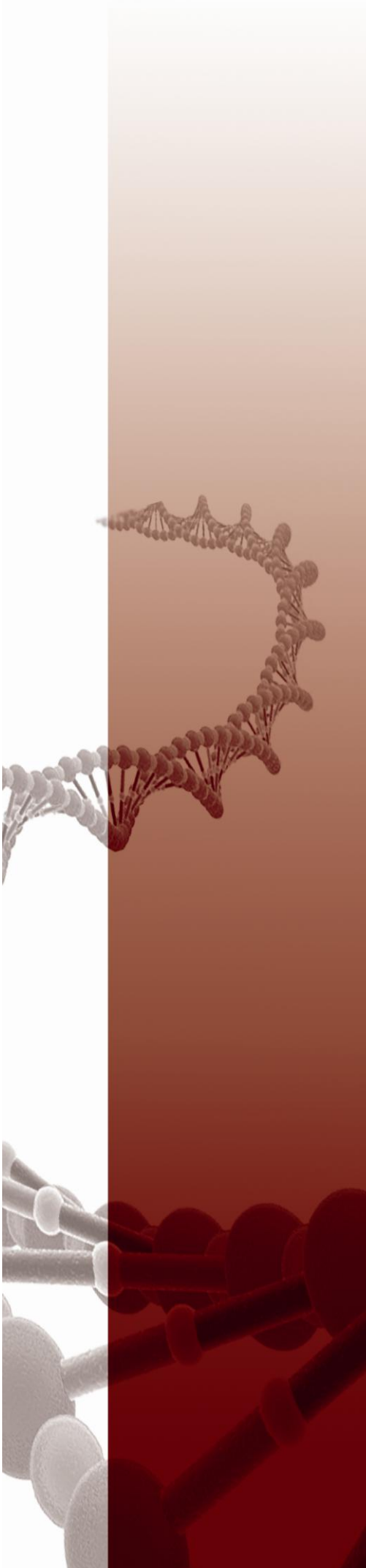
CHAPTER 4: Microbial diversity of anaerobic zones of Tinto River: a culture-dependent and non-dependent approach



INTRODUCTION

The Tinto River is a natural acidic rock drainage (ARD) environment located in the Huelva region, in southwestern Spain. The Tinto River emerges from Peña de Hierro, at the core of the Iberian Pyritic Belt (IPB), one of the largest metal sulfides (pyrite, chalcopyrite, etc.) deposit on Earth, and run to Atlantic Ocean 70 Km far. When exposed to air and water, the microbiologically enhanced oxidation of sulfides produces waters with low pH (pH 2.3) and high ferric iron (~2 g L^{-1}) and sulfate (~6 g L^{-1}) content along the river course. Low pH and ferric iron facilitate metal solubilization; therefore, when these waters come into contact with metallic sulfides, they become highly metalliferous (Cu: ~0.1 g L^{-1}; Zn: ~0.2 g L^{-1}, etc.) (19). Different disciplines have pay attention to the microbiota inhabiting Tinto River: microbiology (ecology of extremophiles living at pH~2), biogeochemistry (exploring the nature of the microbial communities associated with AMD), biotechnology (bioleaching processes), and Mars-analogue studies (jarosite is a mineral common to both Mars and Earth).

Most of the previous work at Tinto River have focused on describing the microbial ecology of the water column (10, 12-14, 22, 35), the diversity associated to macroscopic growths (10) and the eukaryotic diversity (2-4), all of them corresponding to the oxic part of the river. Our group has recently started an in depth Tinto River sediments study to understand how the element cycles work under anaerobic conditions. In that line an extensive survey of the microbial diversity and abundance have been done in two physichochemically contrasting dams (29, 30). Anaerobic activities such as methanogenesis, sulfate-reduction, hydrogen-production and denitrification have been reported (31, 33). Additionally, a study of the microbial diversity associated to the sediments along the river iron gradient (11) have been made.

In this study, we complete the microbial diversity of the sediments -increasing the cloning effort and the sampling sites- and combine it with culture-dependent methods –enrichments for iron-reduction, methanogenesis, hydrogen-production and sulfate-reduction- to pool all the latter studies in a comprehensive common discussion at a global scale.

MATERIAL AND METHODS

Sampling

Samples were collected from eight sampling sites spread in both origins of the river: *UMA* (37.716961,6.554031W), TUN (37.725685N,6.55634W) and *AG* (37.724722N, 6.56W); their union close to Nerva: *CEM* (37.699238,6.557379W), *JL* (37.691207N, 6.560587W) and at the middle course: *EST* (37.68832N, 6.563773W), *SAL* (37.669622N, 6.550212W), and *BER* (37.601346 N, 6.549933W) in November 2010 (Fig.1).

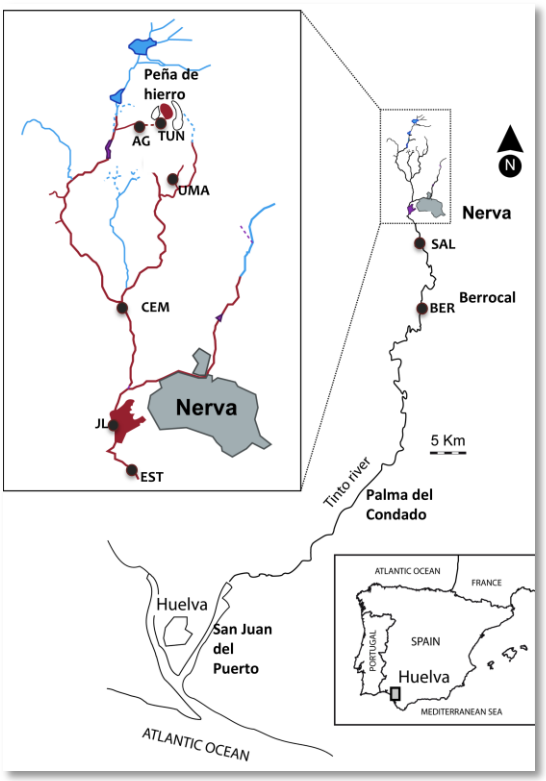


Fig. 1: Map of Tinto River with the sample locations.

The bed of Tinto River is rocky, therefore samples were taken where there was some sediment accumulation pulling a 50 mL-tube into the sediment. Just at *JL* site there is a dam that allows large sediment accumulation, so there, sediment cores (inner diameter, 7 cm; length, 45 cm) were taken with a sampler (Eijkelkamp Agrisearch equipment, The Netherlands). The redox potential (E) and pH of the sediment samples were measured *in situ* with E and pH probes, connected to a Thermo Orion 290A potentiometer placed into the fresh sediment just after extraction. Data about water temperature, conductivity (conductimeter Orion 122, Orion research, USA), pH and redox potential (Ag/AgCl reference pHmeter Crison 506 pH/Eh) and oxygen concentration (Orion 810 oxymeter) were obtained in duplicate in each station.

DNA extraction from sediments and clone library construction.

Total DNA was extracted from 6 g of sediment using FastDNA® SPIN® Kit (for Soil) of Qbiogene, Inc. Adaptation of the commercial protocol was carried out to optimize it for sediments with high concentrations of heavy metals and low biomass content as described before (30), as well as amplification, cloning and sequencing of the 16S rRNA genes. Sequences were assembled using DNABaser program and prior to phylogenetic analysis, vector sequences flanking the 16S rRNA gene inserts were identified using VecScreen tool (NCBI) (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Clone sequences were checked for chimera using Bellerophon tool (18) and compared with the NCBI databases using the Ribosomal Database Project (8) to identify the closest sequence. Sequences were assigned to operational taxonomic units (OTUs) with u-clust method implemented in Qiime (9). Complete sequences (>1400bp) representatives for each OTU were added to a database of over 50000 homologous prokaryotic 16S rRNA primary structures by using the alignment tool of the ARB software package. Phylogenetic reconstruction was performed by using the three algorithms as implemented in the ARB package (23). A consensus tree was generated. The

coverage of the clone libraries was calculated using the equation described by Singleton et al. (17). The PAST software v1.82b (16) was used to compute the statistical indexes and the sampling efficiency in clone libraries of each sampling station. The 16SrRNA genes sequences determined in this study have been deposited in the GenBank database under accession nu - .

Enrichment cultures

Enrichments were prepared in an anaerobic chamber. For all the sampling sites, approximately 3 g of sediment were added to 22 mL glass serum bottles with 20 mL of media and capped with butyl rubber stoppers. The headspace was then degassed with a N_2/CO_2 (80:20) mixture. Enrichments were incubated at 30°C in the dark. The media used were selective for various microbial activities. For methanogenesis, the basal medium described by Sanz *et al* (32) was used at pH 6 supplemented with a mixture of (g L^{-1}): 0.7 sodium formate, 0.5 sodium acetate, 0.3 sodium propionate, 0.3 sodium butyrate, 0.5 methanol, 2 sucrose and 0.7 sodium lactate. For sulfate reduction, the medium used was described previously (31) and used buffered at pH 7 and non-buffered. Enrichments for denitrification were conducted preparing the media as described elsewhere (24). The media used for iron-reduction was (g L^{-1}): 13.7 Fe(III)citrate, 2.5 $NaHCO_3$, 1.5 NH_4Cl, 0.1 KCl, 0.1 yeast extract, 1 sucrose, 3.16 $Na_2S_2O_3{\cdot}5H_2O$, and 1 mL of micronutrients solution (32). L-Cysteine was used as reducing agent (0.05%) in all media.

Banded *JL* dam sediments were analyzed deeply; therefore some methodological differences regarding the rest of the sediment enrichments were made. Approximately 50 g of sediment were added to 122 mL-glass serum bottles with 100 mL of media and capped with butyl rubber stoppers. Two kinds of denitrifying enrichments were conducted separately. Both the heterotrophic (24) and the autotrophic (28) denitrifying media were prepared as described previously. The sulfate-reducing media added contained no buffers to allow the media to equilibrate with the natural pH of the sediment. It was tested with lactate (2 g L^{-1}), hydrogen (gas phase H_2:CO_2 80:20) and nothing (native) as electron donors. Regarding the methanogenic enrichments, they were performed with the mixture described above and also with hydrogen under a H_2/CO_2 (80:20) atmosphere and acetate (0.5 g $L^{-1)}$ as electron donors.

Analytical Measurement

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

reaction of H_2S produced by sulfate reduction and Fe^{2+} added in the media.

Sulfate concentration was determined by ion chromatography with a suppressed conductivity detector (790 Personal IC with an A Supp 5 250/4.0 column Omega Metrohom, Switzerland). Measurements for pH were obtained with an Orion 2-Star portable pH meter (USA). Iron species (ferric, ferrous and total iron) were determined by reflectrometry (Iron test method 1.16983.0001, Reflectoquant, Merck, Germany).

Denaturing gradient gel electrophoresis (DGGE) from sediment samples

DGGE analyses were carried out in *JL* enrichments to get an insight of the responsible organism for each activity. DNA was extracted as explained before for the environmental sediment samples. The V3 to V5 variable regions of the 16S rRNA gene were amplified with the primers set 341F (GC)-907R (annealing temperature (Ta=52°C) for *Bacteria* and 622F (GC)-1100R (Ta =42°C) for *Archaea*. Primers 341F (GC) and 622F (GC) included a GC clamp: 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCC CCG CCC-3' (25). The amplification reaction was performed according to the *Taq* DNA Polymerase protocol (Promega Madison, Wis). The PCR conditions were as follow: 10 min of initial denaturation at 94°C and 30 cycles at 94°C for 1 min and annealing at 52/42°C for 1 min and 72°C for 2 min, followed by 10 min of final primer extension. DGGE analysis was carried out using a D-Code Universal Detection System instrument (Bio-Rad) and a model 475 gradient former according to the manufacturer's instructions (Bio-Rad). Polyacrylamide (6%; 37.5:1 acrylamide-bisacrylamide) gels with a 30 to 60% urea-formamide denaturant gradient (100% urea-formamide contains 7 M urea and 40% deionized formamide) were used in 1x TAE (Tris-acetate-EDTA) buffer, pH 7.4, at 200 V for 4 h at 60°C. Gels were stained with ethidium bromide and visualized under UV illumination. About 100 bands were cut from the gel with a sterile blade and placed in sterile vials with 100 µL of Milli-Q water. DNA was allowed to diffuse into the water at 4 °C overnight. Five microliters of the eluate were used as a DNA template in a PCR of 50 µL with the primers described above but without the GC clamp.

Scanning electron microscopy (SEM)

Sediment samples were studied by SEM as described elsewhere (5). Samples were fixed by immersion in glutaraldehyde (2.5%) for 2 h, washed twice in sodium cacodylate buffer (0.2 M, pH 7.1) and dehydrated in a graded series (10, 30, 50, 70, 90, and 100%) of ethanol/water mixtures for 20 min each. After dehydration, the samples were critical point dried and mounted on stubs. After gold shadowing, the samples were examined in a Phillips XL30 EDAX DX4i SEM.

RESULTS

Physicochemical parameters of the sampling sites

Table 1a and 1b show that in general all sampling sites present a pH range between 1.6 and 2.8. The redox potential (E) ranged between 351.9 and 482.8 mV, positive and high, held to the presence of a strong oxidant, Fe^{3+}. Both, pH and E, are quite constant along the river due to the buffering effect of the Fe^{3+} present in Tinto waters (equation 1).

$$Fe^{3+} + 3\ H_2O \text{ <--> } Fe(OH)_3 + 3H^+ \qquad \text{(eq. 1)}$$

Table 1a: Water physichochemical parameters measured in most of the samples.

Sample	pH	E (mV)	Cond (mS cm^{-2})	SO_4^{2-}	Fe	Mn (mg L^{-1})	Co	Ni	Cu	Zn	As
BER	2.4	482.8	2.8	719.1	280.5	8.2	0.2	0.2	21.8	21.4	0.5
EST	2.2	456.9	8.0	2558.0	1212.6	33.9	1.1	0.5	117	93.9	4.3
CEM	2.4	351.9	9.4	4394.2	1049.7	57.9	3.8	1.1	223	182	2.4
SAL	2.3	418.2	8.2	2754.1	1510.4	37.0	ND	0.6	110	105	5.8
UMA	1.6	459.7	24.8	6861.3	8945.9	10.8	ND	ND	43.9	72.3	15
TUN	2.4	308.9	5.7	1229.8	685.3	13.3	ND	0.0	10.4	40.8	2.0
AG	2.4	459.7	6.1	1815.0	1189.8	18.5	ND	0.1	14.8	64.8	2.0
JL	2.6	356.0	9.3	1738.2	912.6	26.1	ND	0.1	123	92.2	3.1

Conductivity values were the most variable parameter and have a direct relation with metal concentration, especially with iron (the most abundant). Iron concentration decreased along the river, the addition of neutral tributaries has a direct dilution effect lowering the concentrations of metals, and an indirect effect on ferric oxides precipitation that releases protons maintaining a constant pH. The highest values are found at *UMA* (8.9 g L^{-1} of iron and a conductivity of 24.7 mS cm^{-2}) and the lowest at *BER* (0.3 g L^{-1} of iron and a conductivity of 2.8 mS cm^{-2}). The measured sulfate concentrations positively correlated with the iron concentration along the river, logical taking into account that both are the products of the metallic sulfides oxidation.

Table 1b: Water physichochemical concentration (mg L^{-1}) parameters measured in most of the samples.

Sample	acetate	propionate	formiate	nitrite	nitrate	sulfate
BER	0.044	0	0.025	0.256	0.252	558.02
EST	0.177	0	0.025	0.284	0.397	2719.17
CEM	0.796	0.164	0	0.27	0.621	3473.5
SAL	0.044	0	0.027	0.438	0.349	2182.51
UMA	0	3.288	0	0.467	0	7160.53
TUN	0.068	0.1	0	0.12	0	562.4
AG	0.043	0	0.028	0.098	0	733.236
JL	0.469	0	0.102	0.103	0.182	1341.1

Volatile fatty acids and nitrogen species like nitrite and nitrate were detected (Table 1b). It is the first time such data is reported in the Tinto water column. This has important ecological implications showing that substrates and intermediates for denitrification and carbon cycle-related metabolism are present.

With the exception of JL, where real sediment stratification exists, the values of water layer are closed to the values of its underlying sediments. At *JL* sediments, the pH ranged from 3.6 to 5.5 and the redox potential (E) between -141 and 128 mV showing strong variations with depth (Table 2). According to physico-chemical parameters and visual appearance, the JL dam core was composed of two main layers. The reducing black layers (15-25 cm and 40-45 cm) with mean pH of 5.5 and the oxidant brown layers (5-10 and 30-35 cm) with mean pH of 4.5 were excised and immediately stored in 15 mL-tubes at 4°C until further processing in the laboratory (less than one week) for enrichments.

Table 2: Physicochemical parameters measured in *JL* sediment layers.

Depth (cm)	pH	E (mV)
5	3.6	128
10	3.78	106
15	4.5	-60
20	5.5	-118
25	4.7	-47
30	4.3	45
35	4.2	26
40	4.7	-60
45	5	-141

Bacterial clone library diversity. Diversity indexes, coverage, and rarefaction analysis of the 16S rRNA gene libraries.

Former studies of the Tinto Sediments (11, 14, 30) coincided in their low archaeal diversity, falling all the sequences retrieved in the *Thermoplasma* class. Therefore, archaeal community was not further investigated in this study. Positive amplifications were obtained with bacterial specific primers in all sediments samples. A total of 864 clones were obtained. After elimination of chimerical and bad quality sequences, 714 sequences were analyzed.

All data analysis was performed for the total set of sequences and in each clone library separately. Globally, the 714 total sequences clustered in 176 OTUs, based on 97% sequence similarity (27). Specifically per sample, the number of OTUs varied from 46 till 92 (Table 2). Between the 176 OTUs, 29 OTUs appeared to be common at different sampling sites and others exclusive for one. There were some remarkable OTUs, which appear widely in the clone libraries. For instance, clones related to the actinobacterial genus *Ferrimicrobium* were detected in *UMA*, *TUN*, *AG* and *CEM* (OTU 10). This genus corresponds to heterotrophic, iron-oxidizing and extremely acidophilic actinobacteria (20).

The common iron oxidizers in ARD environments are members of the aerobic chemolithotrophic bacterial genus *Leptospirillum* (*Nitrospira*), which appear in different locations (OTU 45 in *CEM* and *AG* and OTU 94 in *TUN* and *EST*) and the facultative anaerobe gammaproteobacterial *Acidithiobacillus ferrooxidans,* which can oxidize iron and sulfur in aerobic conditions and reduce iron in anoxia and appeared in most of the samples (OTU 120 in *TUN*, *CEM*, *SAL* and *BER*; OTU 135 in *TUN*, *CEM* and *UMA* and OTU 171 in *TUN*, *BER*, *CEM*, *EST* and *SAL*). In the Tinto River, together with the two former genera mentioned above, the alphaproteobacterial *Acidiphilium* also appear at different locations (OTU 24 in *EST* and *CEM*; OTU 147 in *UMA*, *EST*, *CEM* and *SAL*). This heterotrophic bacterium is able to oxidize organic compounds using ferric iron as electron acceptor, even in the presence of oxygen, playing a key role in the mineralization of organic matter, toxic at low pH for the acidophiles.

Within the phylum *Firmicutes*, several clones clustering within *Clostridiales* were retrieved from *JL black*, *EST* and *BER* (OTU 170) and the sulfate-reducing *Desulfosporosinus* spp. were found in *JL black* and *TUN* (OTU 1). Deltaproteobacterial clones belonging to the sulfur-reducer *Desulfurella* genus were identified exclusively in both layers of *JL* dam (OTU 17).

Moreover, sequences related to the genus *Dechloromonas* were obtained at *BER*, *AG*, *SAL*, *UMA* and *CEM* (OTUs 57 and 112). They are metabolic versatile organisms that can oxidize aromatic compounds such as toluene, benzoate, and chlorobenzoate or Fe(II) and AHDS (a reduced humic compound) aerobically and anaerobically using nitrate or even perchlorate or chlorate

as a suitable electron acceptor (1, 7). Therefore, they have a great potential for bioremediation processes.

Table 3: Statistical indexes in each sample

Sample	*BER*	*EST*	*CEM*	*SAL*	*UMA*	*TUN*	*AG*	*JL black*	*JL brown*	TOTAL
N° clones	96	96	96	96	96	96	96	96	96	864
N° sequences	79	81	85	92	89	78	46	85	79	714
N° OTUs	39	24	30	21	39	44	31	37	12	175
Good´s coverage value (%)	25.6	54.2	60	61.9	35.9	34.1	25.8	32.4	58.3	44.9
Shannon-Wiener index	3.18	2.8	3.01	2.68	3.28	3.50	3.23	3.11	1.65	4.32
Dominance (D) value	0.07	0.08	0.06	0.08	0.05	0.04	0.05	0.07	0.3	0.02

Rarefaction curves, Good's coverage values and diversity indexes were calculated separately for each sample site. Good's value (Table 3), an estimation of the proportion of the population represented by the retrieved sequences (15), indicates low coverage of the bacterial libraries, in agreement with the rarefaction curves (Fig. 2).

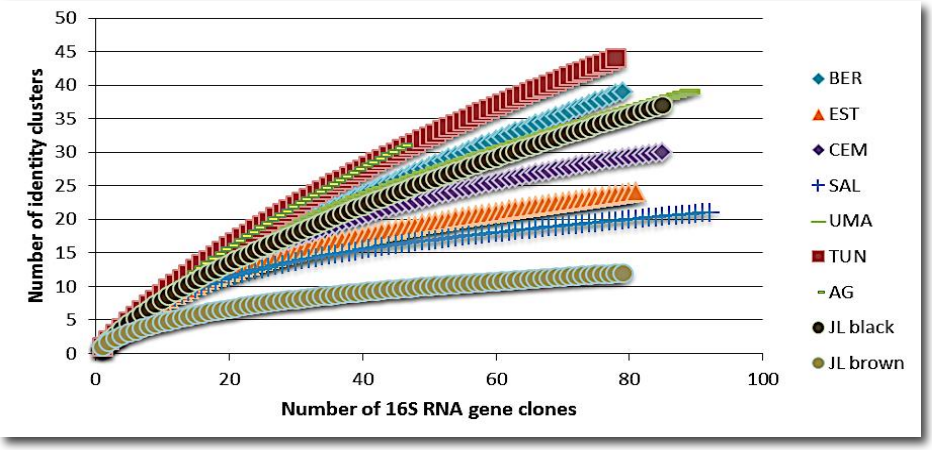


Fig. 2: Rarefaction curves for each sample.

Dominance (D) values are very low in all samples sites and in the global analysis, close to 0 (no OTUs predominance in the community), with exception of JL Brown (D=0.3) indicating the predominance of some OTUs in JL brown sequence data set (*Desulfurella* clones). In accordance with this, the Shannon-Wiever index value was lower in *JL* Brown than in the rest of samples. Higher diversity indexes were obtained compared with previous studies in Tinto River (30).

It was possible to identify phylogenetically down to the genus level most of the *Bacteria* sequences. 61 genera were identified, falling in twelve different phyla: *Cloacibacillus* (*Synergistetes*); *Bacillariophyta* (*Cyanobacteria/Chloroplast*); *TM7*_incertae_sedis (*TM7*); family *Anaerolineaceae* (*Chloroflexi*); *Leptospirillum* (*Nitrospira*), *Prosthecobacter* (*Verrucomicrobia*); *Fibrobacter* (*Fibrobacter*); *Alkalibacterium*, *Bacillus*, *Paenibacillus*, *Oceanobacillus*, *Staphylococcus*, *Tumebacillus*, *Alicyclobacillus* (*Bacilli* class, *Firmicutes*); *Turicibacter*

(*Erysipelotrichia* class, *Firmicutes*); *Thermodesulfobium*, *Desulfosporosinus*, *Desulfotomaculum*, *Oxobacter*, *Anaerobacter*, *Clostridium* sensu stricto, *Clostridium* XI, *Clostridium* IV, *Dorea*, *Sulfobacillus* (*Clostridia* class, *Firmicutes*), *Olsenella*, *Ferrimicrobium*, *Ferrithrix*, *Aciditerrimonas*, *Rothia*, *Brevibacterium*, *Leucobacter*, *Propionibacterium* (*Actinobacteria*); *Acidiphilium*, *Sphingomonas* (*Alphaproteobacteria*); *Methylotenera*, *Aquabacterium*, *Ideonella*, *Acidovorax*, *Zooglea*, *Ferribacterium*, *Dechloromonas* (*Betaproteobacteria*), *Legionella*, *Steroidobacter*, *Pseudomonas*, *Cellvibrio*, *Enhydrobacter*, *Acinetobacter*, *Acidithiobacillus* (*Gammaproteobacteria*), *Geobacter*, *Desulfurella*, *Desulfosalsimonas*, *Desulfobulbus* (*Deltaproteobacteria*), *Flavobacterium*, *Fluviicola*, *Haliscomenobacter*, *Flectobacillus*, *Leadbetterella*, *Pedobacter*, *Paludibacter* (*Bacteroidetes*), *Anaerolineaceae* family (*Chloroflexi*) and subgroups *Gp1*, *Gp3* (*Acidobacteria*). The distribution of the different phyla varied between the different sample sites (Fig. 3).

A big amount of sequences correspond to groups never detected before in the Tinto ecosystem. Taking in account the bacterial diversity found in previous studies (11, 30) and this work, the results evidence that there is a high microbial diversity inhabiting the anaerobic zones of Tinto basin.

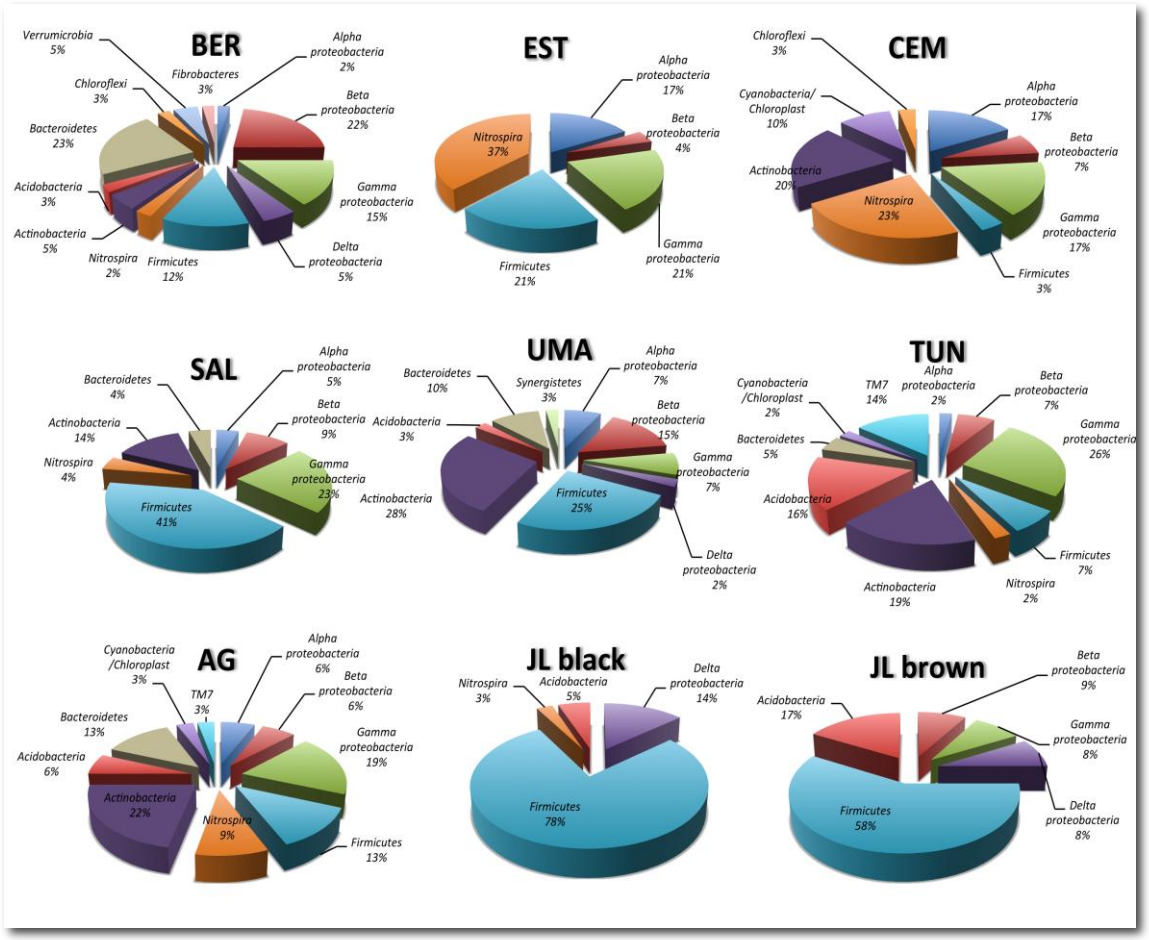


Fig. 3: Phylogenetic composition of the bacterial 16S rRNA gene libraries of the nine samples: *BER*, *EST*, *CEM*, *SAL*, *UMA*, *TUN*, *AG*, *JL* Black and *JL* Brown.

Anaerobic Activities Screening

Methanogenesis, denitrification, iron-reduction and sulfate-reduction were assayed at all sampling sites. *JL* enrichments will be explained separately in more detail as they were studied deeply. Table 4 shows a qualitative summary of the measured activities at the different sampling sites and Figure 4 shows the visual changes of the enrichments.

Table 4: Summary of anaerobic activities and sampling sites.

	BER	*EST*	*CEM*	*SAL*	*UMA*	*TUN*	*AG*
Methanogenesis	-	-	+	-	-	+	-
Denitrification	+	+	+	++	-	+	+
Iron-reduction	+	++	+	-	+	+	++
Sulfate-reduction pH <5	-	-	-	+	-	-	-
Sulfate-reduction pH ~7	+	+	+	+	-	+	+

Methanogenesis just took place in two of the enrichments showing both low methane production (pmol g^{-1} day^{-1}): *CEM* (9) and *TUN* (4). In contrast all the sampling sites yield positive results for denitrification (except *UMA*) and iron-reduction (except *SAL*). As observed in figure 4, the reduction from Fe^{3+} (red) to Fe^{2+} (green) can be easily detected visually (the negative control is at the top of the enrichments). It can also be observed a green coloration in the denitrifying enrichment, which correlates with N_2O production, an intermediate of nitrate-reduction. This marking (greenish coloration) was previously observed in Tinto River enrichments (31) and it is probably due to the presence of polysulfides, which produce a green coloration (6), formed in the oxidation of sulfide coupled to the denitrification. Regarding the sulfate-reduction, activity was determined visually by the formation of black precipitates from the reaction of S^{2-} produced by sulfate reduction and Fe^{2+} added in the media. The un-buffered media for sulfate-reduction just allow activity in the enrichment from *SAL*, starting at a pH 4.5 and increasing to 6.5. All the enrichments, except *UMA*, showed sulfate-reduction with the buffered media at pH 7. Remarkably, UMA did not showed activity in the specific media for sulfate-reduction but black FeS precipitates could be observed in the iron-reduction-specific media, suggesting sulfate-reduction activity in the enrichment.

These results showed a diverse distribution of different anaerobic activities such as methanogenesis or sulfate-reduction, which normally take place at reducing and neutral pH in a strongly acid and oxidant bulk environment (pH 2.3; E +400mV).

Fig. 4: Screening of anaerobic activities at the different sampling sites.

Anaerobic Activities at different pH.

(i) Activities rates

Methanogenesis was investigated in both layers of *JL* using hydrogen, acetate and a complex mixture of VFA as electron donors. The methane production was rather slow in general averaging 1.5 pmol CH_4 per gram wet sediment and day except in JL black biostimulate with the mixture M that produce 13 pmol CH_4 per gram wet sediment and day. The visual observation of the methanogenic enrichments (Fig. 5) evidenced the simultaneous occurrence of sulfate-reduction in most of the bottles by the presence of FeS (black) and Fe_2S (metallic) precipitates.

Denitrifying enrichments were performed with sediments from reducing (black) and oxidant (brown) layers using heterotrophic and autotrophic media. Denitrification activity was followed by nitrate (NO_3^-), reduction and nitrite (NO_2^-) and nitrous oxide (N_2O) production. All the enrichments showed activity at the end of the experiment (61 days) but heterotrophic ones were faster showing activity at day 15. Nitrate was consumed during the assay with an average (µmol g^{-1} day^{-1}) of 2.6 for black layer-enrichments and 1 for brown layer-enrichments showing a better develop at higher pH. The nitrous oxide production measured (µmol g^{-1} day^{-1}) was also higher in black (1) than in brown (0.7) layer. Comparing the nitrous oxide production with the nitrate reduction rates, it was seen that a 37% of the reduced nitrate is accumulated in nitrous oxide with the black layer inoculum while the 69% is accumulated with the brown layer inoculum. The optimum pH range for complete reduction of nitrate to nitrogen gas (N_2) is considered to be between 6 and 8; below this optimal pH range, it has been reported that final steps of the denitrification are inhibited and the proportion of intermediate products, as nitrous oxide, increases with respect to dinitrogen gas (34). This result is in accordance with previous ones performed with Tinto river sediments (31). Positive activity correlated with the appearance of green colour in the bottles even in a quantitative way.

Iron-reduction activity was studied for both layers. The reduction of ferric iron results higher at lower pH, reaching till 75% of the added iron at day 15 in brown-layer enrichment while in black-layer enrichment took 61 days to achieve 50% reduction. In figure 5 it can be observed that after iron-reduction took place, sulfate-reduction started, reasonable taking in account suggesting that high Fe concentrations would inhibit sulfate-reduction.

Finally, sulfate-reduction carried-out by both layers was investigated with un-buffered media using hydrogen and lactate as electron donors and a control with the natural donors of the sample. The results showed clearly that sulfate-reduction takes place better at pH around 5 (black layer) than at pH around 4 (brown layer). In just 3 days the sulfate-activity could be observed in all the enrichments from black layers. At day 61, enrichments of brown layer with hydrogen and natural turned positive while enrichment with lactate remained negative, confirming the inhibition caused for organic acids at pHs below its pKa. This result showed the sulfate-reduction inhibition with the organic acid lactate at pH 4 while there was no inhibition with hydrogen at pH as low as 3.6.

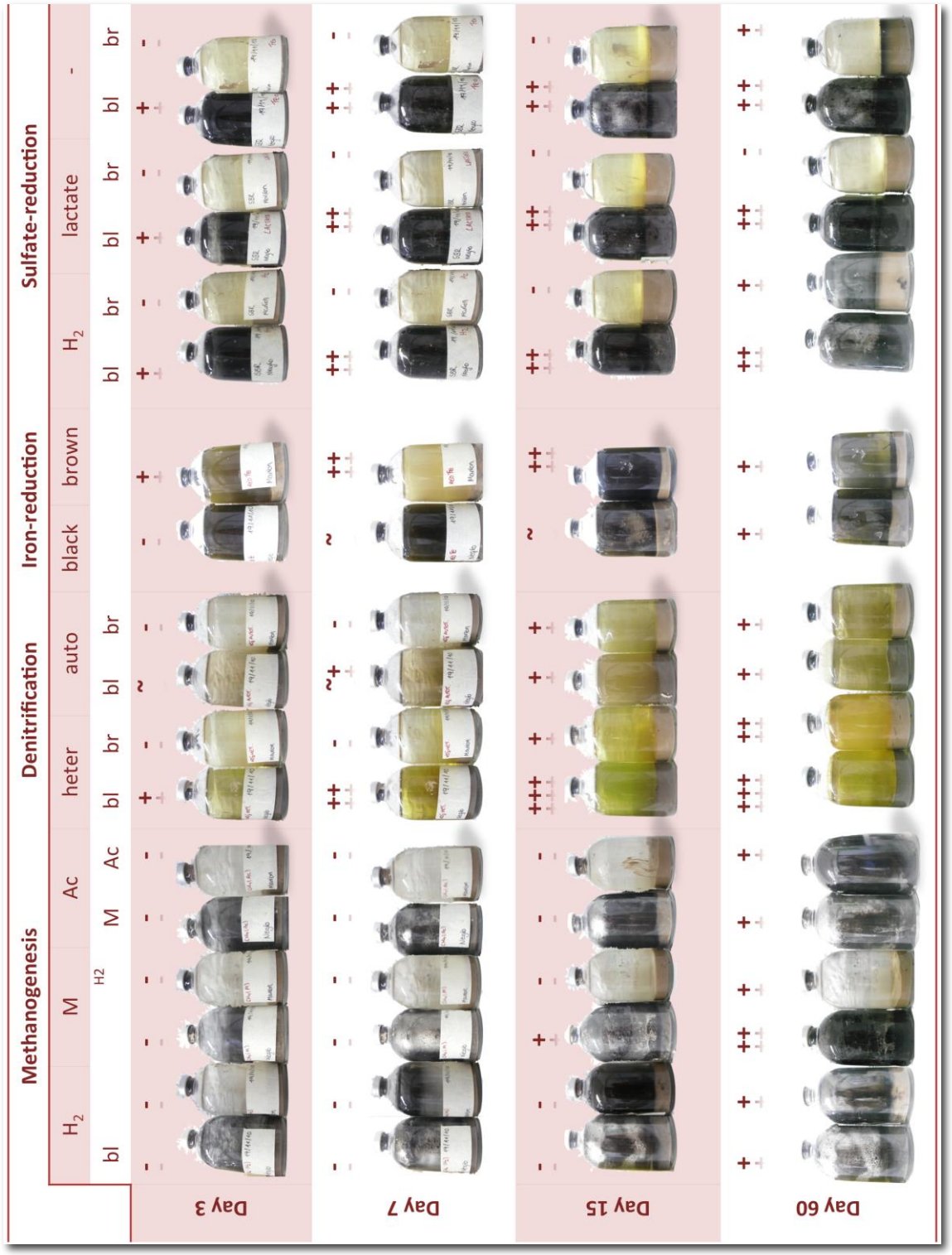


Fig. 5: Scheme of *JL* enrichments.

(ii) DGGE fingerprint analyses of JL enrichments

Microbial populations enrichments were analyzed by DGGE to reveal the diversity and taxonomic position of the responsible microbes. The bacterial fingerprint (Fig. 6a) showed a relatively high number of bands in the sediments used as inocula, in accordance with previous studies of microbial diversity at *JL* sediments (30). The pattern of bands differed more between the different activities than between different inocula. This suggests that the

conditions will force the development of some microorganism over another, taking place some metabolisms, but between different pH conditions, the microorganism would be similar but behave different in dependence of their optimal conditions.

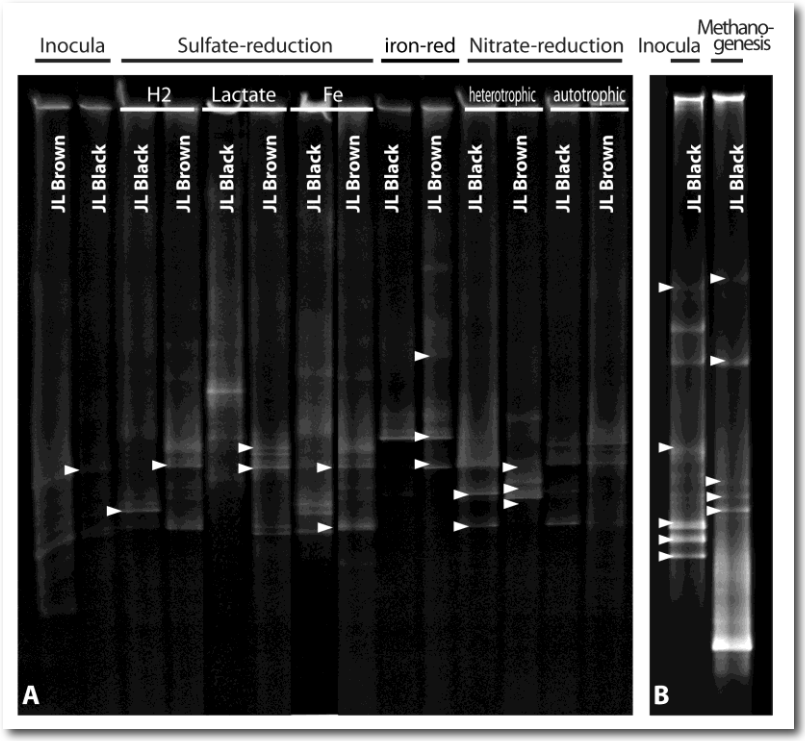


Fig. 6: DGGE fingerprints of bacterial (a) and archaeal (b) sequences retrieved from the JL enrichments. Arrows represent the bands that yield quality sequences and were deposit in GenBank database.

Sequences from sulfate-reduction enrichments belonged to genus *Bacteroides* within phylum *Bacteroidetes* (JQ815575, natural donors), genus *Clostridium* (JQ815572/3, lactate as donor) and genus *Desulfosporosinus* (JQ815576, natural donors and JQ815574, lactate as donor) within phylum *Firmicutes*. *Desulfosporosinus* is a known sulfate-reducing bacteria found in Tinto River samples and enrichments. Bacteria sequences from iron-reduction enrichments clustered in the family *Bradyrhizobiacea* (JQ815577) within *Alphaproteobacteria* class related with an iron-reducing clone (FJ802302) and *Clostridium* genus (JQ815578). Sequences retrieved from denitrifying enrichments clustered in genera *Alicyclobacillus* (JQ815580/81/82) and *Desulfurella* (JQ815579).

Archaeal sequences were amplified from the only enrichment that produced methane significantly (JL black with mixture M as electron donor) and from the corresponding sediment used as inocula. All the archaeal sequences of the methanogenic JL black culture clustered in *Methanomicrobiota* class (*Euryarchaeota* phylum) sharing high similarity with *Methanosaeta thermophile*. The amplification of the inoculum used (JL black sediment) showed sequences belonging to *Thermoplasmatales* order (JQ815588/92), as usually found in Tinto River sediments. Remarkably *Methanosaeta concilii* (JQ815591) was identified in the sediment used as inocula.

Scanning electron microscopy (SEM)

SEM analysis of sediments revealed a general scarce colonization of the analyzed sediments (Fig.7). This corroborated the general trend observed previously (29). Different prokaryotic

morphologies could be observed as well as diatoms and fungus structures.

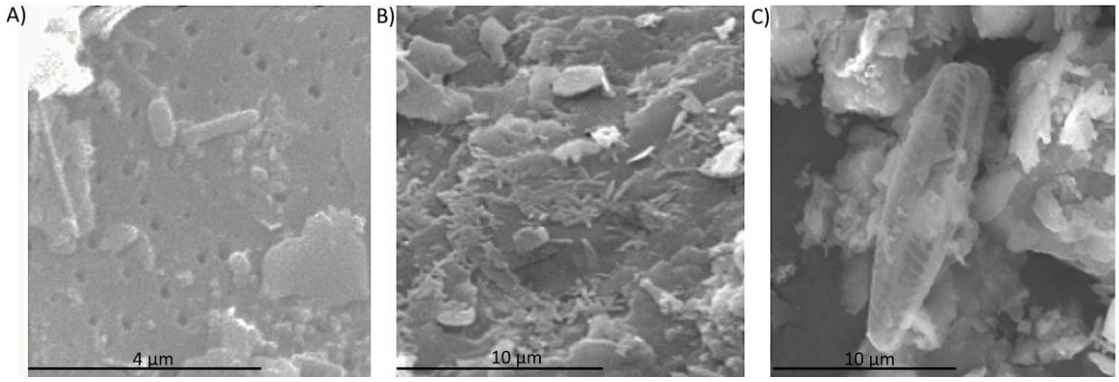


Fig. 7: SEM images of the sediments.

DISCUSSION

The Tinto River looks quite homogeneous looking at parameters as pH (2.3±0.4) or redox potential (419±61) showing acid and oxidant conditions along the course. But due to the hydrology, e.g. addition of neutral tributaries, the river presents a metal gradient along its course, specially notary for iron (g L^{-1}) ranging from 8.9 at the upper part till 0.3 in the middle course (with the consequent conductivity variation), but also important in outstanding for most of the metals: Cu (88.7±69), Co (3.8±3.8), Mn (27.7±16), and metalloids: As (5±4.6).

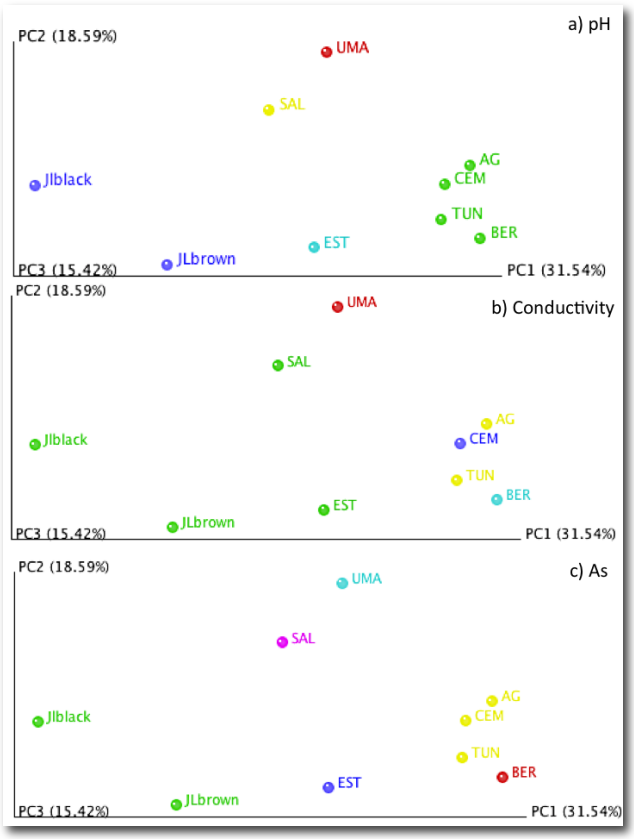


Fig. 8: Statistical analysis performed with FastUniFrac attending to different physicho-chemical parameters such as pH (a), conductivity (b) and arsenic concentration (c).

Playing attention to the samples distribution with specific characteristics, statistical analysis shows that some parameters influence on microbial distribution. When analyzing the distribution according to the pH values, it can be seen in Figure 8a that *AG*, *SEM*, *TUN* and *BER*, which clustered together, share common pH values. In the same way conductivity bring together *SAL*, *EST* and *JL*, which shared medium values (Fig. 8b). Remarkably arsenic also showed a weighting effect on the samples distribution that clustered *AG*, *TUN* and *CEM* (Fig. 8c).

The microbial diversity of Tinto River water is limited to a few organisms (14). Microbial consortia in AMD waterflows are generally dominated by acidophilic, autotrophic iron- or sulfur-oxidizing prokaryotes such as members of *Acidithiobacillus* and *Leptospirillum* genera and heterotrophic acidophiles as *Acidiphilium* (21). But studies in two dams previously studied showed an extensive biodiversity (30, 31), what it is definitely confirmed with the results of this study in anaerobic zones through the river.

The superficial sediments layers would act as transition from the oxic water overlayed characteristics to the anaerobic deeper sediments. Therefore in this zone, we can find organisms as *At. ferrooxidans* and *L. ferrooxidans*, specially adapted to the high ionic strength and oxidative stress (26) present in Tinto waters but also a complex community which links iron, sulfur, nitrogen and carbon cycles with intricate relationships. The development of anaerobic conditions will favor sequentially different anaerobic activities as correspond to their redox chemistry. First of all, the reduction of the ferric iron would occur, what will turn the redox potential less oxidant allowing the occurrence of nitrate reduction. Denitrification, in general, increases the pH favoring less extreme conditions in the sediments (31). Next, metabolism like the sulfate-reduction would have place increasing the pH and reducing the redox potential (29). And lately, with a very low energy yield, the methanogenesis would occur (33).

In the cloning of environmental sediment samples and supported by the DGGE sequences retrieved from enrichments, we have found sequences belonging to organism able to develop the different anaerobic activities studied and detected. Microorganisms involved in the reduction of iron (*A. ferrooxidans*, *Sulfobacillus*, *Geobacter*, *Acidiphilium, Acidobacteria…)* were identified as well as sulfate-reducers (*Desulfurella*, *Desulfosporosinus*, *Desulfotomaculum*, *Desulfobulbus* and *Thermodesulfobium*). Nitrate-reducers were widely identified: *Dechloromonas*, *Propionibacterium*, *Pseudomonas*, *Bacillus…* Methanogens, just identified by DGGE, correspond to *Methanosaeta* spp. Besides the activities tested, another activities, as fermentation, can be inferred from the clone libraries (*Clostridium* and *Bacillus and related species*). The results of this study clearly highlight the importance of this compartment as a cluster where an active iron, sulfur, nitrogen, carbon and metals cycles are operating.

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OTU ID	AG	BER	CEM	EST	JLblac	JLbro	SAL	TUN	UMA	Consensus Lineage
1					4			1		*Firmicutes;Clostridia;Clostridiales;Peptococcaceae;Desulfosporosinus*
2					1					*Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Oxobacter*
3								1		*Actinobacteria;Actinobacteria;Acidimicrobiales*
4			1						1	*Proteobacteria;Betaproteobacteria;Methylophilales;Methylophilaceae*
5								1		*Bacteroidetes;Flavobacteria;Flavobacteriales;Cryomorphaceae;Fluviicola*
6								1		*TM7;TM7_genera_incertae_sedis*
7			1							*Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae;Acidiphilium*
8					2					*Firmicutes;Clostridia;Clostridiales;Peptococcaceae;Desulfosporosinus*
9									3	*Bacteroidetes*
10	3		9					1	5	*Actinobacteria;Actinobacteria;Acidimicrobiales;Acidimicrobiaceae;Ferrimicrobium*
11							3			*Firmicutes;Bacilli;Bacillales;Bacillaceae;Oceanobacillus*
12							3			*Firmicutes;Bacilli;Bacillales;Bacillaceae;Oceanobacillus*
13		2								*Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Acidovorax*
14		1								*Firmicutes;Clostridia;Clostridiales;Incertae Sedis XVII;Sulfobacillus*
15		1								*Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae;Leptospirillum*
16		1								*Firmicutes;Clostridia;Clostridiales;Ruminococcaceae*
17					1	41				*Proteobacteria;Deltaproteobacteria;Desulfurellales;Desulfurellaceae;Desulfurella*
18							2			*Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae*
19	1		1							*Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae;Acidiphilium*
20	1									*Proteobacteria;Betaproteobacteria*
21								1		*Proteobacteria;Gammaproteobacteria;Chromatiales*
22							8			*Actinobacteria;Actinobacteria;Actinomycetales*
23								1		*Bacteroidetes;Sphingobacteria;Sphingobacteriales;Cytophagaceae;Flectobacillus*
24			1	2						*Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae;Acidiphilium*
25					1					*Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobacteraceae*
26		1								*Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae*
27								1		*Proteobacteria;Gammaproteobacteria*
28								1		*Proteobacteria;Deltaproteobacteria*
29								2		*Acidobacteria;Acidobacteria_Gp1;Gp1*
30					2					*Firmicutes;Clostridia;Clostridiales;Peptococcaceae*
31	1									*Actinobacteria;Actinobacteria;Acidimicrobiales*
32	1									*Proteobacteria;Gammaprot.;Xanthomonadales;Sinobacteraceae;Steroidobacter*
33			1							*Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae*
34	1									*Actinobacteria;Actinobacteria;Acidimicrobiales;Acidimicrobiaceae;Ferrithrix*
35	1		8							*Actinobacteria;Actinobacteria;Acidimicrobiales;Acidimicrobiaceae*
36					1					*Firmicutes;Clostridia;Clostridiales;Ruminococcaceae*

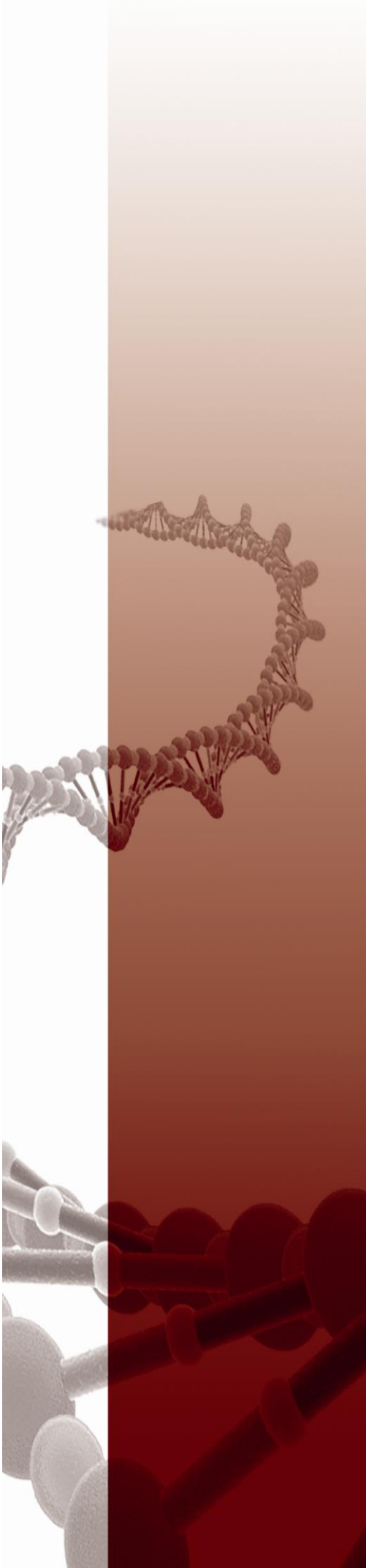
OTU ID	AG	BER	CEM	EST	JLblac	JLbro	SAL	TUN	UMA	Consensus Lineage
37									1	Proteobacteria;Gammaprot.;Pseudomonadales;Pseudomonadaceae;Pseudomonas
38									1	Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus
39							1			Firmicutes;Bacilli;Bacillales;Bacillaceae;Oceanobacillus
40						1				Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;Sporacetigenium
41							1			Bacteroidetes;Sphingobacteria;Sphingobacteriales;Cytophagaceae;Leadbetterella
42				9						Proteobacteria;Gammaprot.;Acidithiobacillales;Acidithiobacillaceae;Acidithiobacillus
43					1					Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium
44									1	Actinobacteria;Actinobacteria;Actinomycetales;Propionibacteriaceae
45	8		29	3	2					Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae;Leptospirillum
46		1								Bacteroidetes;Sphingobacteria;Sphingobacteriales;Sphingobacteriaceae;Pedobacter
47		2								Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Paludibacter
48								1		Acidobacteria;Acidobacteria_Gp1;Gp1
49								2		TM7;TM7_genera_incertae_sedis
50						5				Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;Sporacetigenium
51		1								Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Cellvibrio
52		3								Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;Desulfobulbus
53		1								Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae;Fibrobacter
54								1		Actinobacteria;Actinobacteria;Acidimicrobiales
55		1								Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Dorea
56					1					Firmicutes;Bacilli;Bacillales;Alicyclobacillaceae;Alicyclobacillus
57	1	11	1	1			1	2	10	Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Dechloromonas
58					1					Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium
59					1					Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium
60				1						Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae;Leptospirillum
61						13				Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;Sporacetigenium
62	2									Acidobacteria;Acidobacteria_Gp1;Gp1
63			3					1		Cyanobacteria;Cyanobacteria;Chloroplast;Bacillariophyta
64					1					Bacteria
65			1							Proteobacteria;Gammaproteobacteria;Legionellales;Legionellaceae;Legionella
66					3					Firmicutes;Clostridia;Thermoanaerobacterales;Thermodesulfobiaceae;Thermodesulfobium
67	1									Actinobacteria;Actinobacteria;Acidimicrobiales;Iamiaceae;Iamia
68									1	Proteobacteria;Deltaproteobacteria;Desulfuromonadales;Geobacteraceae;Geobacter
69									1	Proteobacteria;Betaprot.;Burkholderiales;Burkholderiales_incertae_sedis;Aquabacterium
70									1	Firmicutes;Clostridia;Clostridiales;Incertae Sedis XVII;Sulfobacillus
71									1	Bacteroidetes
72									1	Firmicutes;Clostridia;Clostridiales;Incertae Sedis XVII;Sulfobacillus

OTU ID	AG	BER	CEM	EST	JLblac	JLbro	SAL	TUN	UMA	Consensus Lineage
73	1									*TM7;TM7_genera_incertae_sedis*
74					3					*Acidobacteria;Acidobacteria_Gp1;Gp1*
75						3				*Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;Sporacetigenium*
76									1	*Actinobacteria;Actinobacteria;Acidimicrobiales;Acidimicrobiaceae*
77									1	*Actinobacteria;Actinobacteria;Actinomycetales;Micrococcaceae;Rothia*
78	1									*Bacteroidetes*
79							5			*Firmicutes;Bacilli;Bacillales;Bacillaceae;Oceanobacillus*
80							3			*Actinobacteria;Actinobacteria;Actinomycetales;Brevibacteriaceae;Brevibacterium*
81								1		*Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae*
82								1		*Proteobacteria;Gammaproteobacteria;Chromatiales;Chromatiaceae;Nitrosococcus*
83			2							*Cyanobacteria;Cyanobacteria;Chloroplast*
84		5								*Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Acinetobacter*
85									6	*Firmicutes;Bacilli;Bacillales;Bacillaceae;Tumebacillus*
86				6						*Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae;Leptospirillum*
87						1				*Firmicutes;Clostridia;Clostridiales;Incertae Sedis XVII;Sulfobacillus*
88						1				*Firmicutes;Clostridia;Thermoanaerobacterales;Thermodesulfobiaceae;Thermodesulfobium*
89		1								*Actinobacteria;Actinobacteria;Actinomycetales;Microbacteriaceae;Leucobacter*
90		1								*Proteobacteria;Betaprot.;Burkholderiales;Burkholderiales_incertae_sedis;Aquabacterium*
91								1		*Acidobacteria;Acidobacteria_Gp1;Gp1*
92		1								*Verrucomicrobia;*e;Verrucomicrobiales;Verrucomicrobiaceae;Prosthecobacter*
93								1		*Proteobacteria;Gammaproteobacteria*
94	2		4	27			1	1		*Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae;Leptospirillum*
95						1				*Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae*
96							4			*Firmicutes;Bacilli;Bacillales;Bacillaceae;Oceanobacillus*
97									1	*Synergistetes;Synergistia;Synergistales;Synergistaceae;Cloacibacillus*
98							11		2	*Firmicutes;Bacilli;Bacillales;Bacillaceae;Oceanobacillus*
99								1		*Firmicutes;Bacilli;Bacillales*
100								4		*TM7;TM7_genera_incertae_sedis*
101								1		*Actinobacteria;Actinobacteria;Acidimicrobiales*
102					1					*Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium*
103					5					*Proteobacteria;Deltaproteobacteria;Desulfurellales;Desulfurellaceae;Desulfurella*
104					25					*Proteobacteria;Deltaproteobacteria;Desulfurellales;Desulfurellaceae;Desulfurella*
105					2					*Firmicutes;Clostridia;Clostridiales;Peptococcaceae;Desulfotomaculum*
106					1					*Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;Sporacetigenium*
107	1									*Firmicutes;Clostridia;Clostridiales;Incertae Sedis XVII;Sulfobacillus*
108	1									*Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae*

OTU ID	AG	BER	CEM	EST	JLblac	JLbro	SAL	TUN	UMA	Consensus Lineage
109		1								Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae
110									1	Bacteroidetes;Sphingobacteria;Sphingobacteriales;Saprospiraceae;Haliscomenobacter
111		1							1	Acidobacteria;Acidobacteria_Gp3;Gp3
112		1							2	Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Dechloromonas
113		1								Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Paludibacter
114							25			Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Staphylococcus
115		1								Proteobacteria;Betaprot.;Burkholderiales;Burkholderiales_incertae_sedis;Ideonella
116			1							Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae;Acidiphilium
117						2				Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Ferribacterium
118					1					Firmicutes;Clostridia;Clostridiales;Peptococcaceae
119								1		Acidobacteria;Acidobacteria_Gp1;Gp1
120	4	6	1				7	1		Proteobacteria;Gammaprot.;Acidithiobacillales;Acidithiobacillaceae;Acidithiobacillus
121				1	1					Firmicutes;Erysipelotrichi;Erysipelotrichales;Erysipelotrichaceae;Turicibacter
122					1					Firmicutes;Clostridia;Clostridiales;Clostridiaceae
123								8		Acidobacteria;Acidobacteria_Gp1;Gp1
124								2		Proteobacteria;Gammaprot.;Acidithiobacillales;Acidithiobacillaceae;Acidithiobacillus
125								2		Actinobacteria;Actinobacteria;Acidimicrobiales
126							1	14		Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae
127									5	Actinobacteria;Actinobacteria;Acidimicrobiales;Acidimicrobiaceae
128									5	Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus
129				2	1					Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium
130									1	Proteobacteria;Gammaprot.;Pseudomonadales;Pseudomonadaceae;Pseudomonas
131									9	Firmicutes;Bacilli;Bacillales;Bacillaceae;Tumebacillus
132			3							Actinobacteria;Actinobacteria;Acidimicrobiales;Acidimicrobiaceae
133	1									Cyanobacteria;Cyanobacteria;Chloroplast
134		1								Bacteroidetes
135			3					4	1	Proteobacteria;Gammaprot.;Acidithiobacillales;Acidithiobacillaceae;Acidithiobacillus
136								3		Actinobacteria;Actinobacteria
137		1								Bacteroidetes;Sphingobacteria;Sphingobacteriales;Chitinophagaceae;Ferruginibacter
138								1		Firmicutes;Bacilli;Bacillales
139	1							1	2	Actinobacteria;Actinobacteria;Acidimicrobiales
140					1					Firmicutes;Clostridia;Clostridiales;Peptococcaceae
141									2	Actinobacteria;Actinobacteria;Acidimicrobiales;Acidimicrobiaceae;Ferrimicrobium
142		1								Proteobacteria;Alphaprot.;Sphingomonadales;Sphingomonadaceae;Sphingomonas
143								4		Bacteria
144		2								Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Flavobacterium

OTU ID	AG	BER	CEM	EST	JLblac.	JLbro	SAL	TUN	UMA	Consensus Lineage
145				1						Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Enhydrobacter
146								2		Proteobacteria;Betaproteobacteria
147			1	10			1		12	Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae;Acidiphilium
148						4				Acidobacteria;Acidobacteria_Gp1;Gp1
149				1						Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae;Leptospirillum
150									1	Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae
151									1	Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Zoogloea
152	2									Firmicutes;Clostridia;Clostridiales;Incertae Sedis XVII;Sulfobacillus
153	2		2							Firmicutes;Clostridia;Clostridiales;Incertae Sedis XVII;Sulfobacillus
154	1									Actinobacteria;Actinobacteria;Acidimicrobiales
155		1								Verrucomicrobia;*e;Verrucomicrobiales;Verrucomicrobiaceae;Prosthecobacter
156		4								Bacteroidetes
157		3								Chloroflexi;Anaerolineae;Anaerolineales;Anaerolineaceae
158								1		Bacteria
159		1			1					Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;Peptostreptococcus
160	1									Bacteroidetes;Sphingobacteria;Sphingobacteriales;Chitinophagaceae
161	1									Acidobacteria;Acidobacteria_Gp1;Gp1
162									1	Firmicutes;Bacilli;Lactobacillales;Carnobacteriaceae;Alkalibacterium
163						5				Acidobacteria;Acidobacteria_Gp1;Gp1
164									1	Firmicutes;Bacilli;Bacillales;Paenibacillaceae;Paenibacillus
165						1				Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Anaerobacter
166					3					Firmicutes;Clostridia;Clostridiales;Peptococcaceae;Desulfosporosinus
167									1	Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus
168	1								1	Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Flavobacterium
169	1									Bacteroidetes
170		2		4	16		1			Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae
171	2	3	10	6			14	4		Proteobacteria;Gammaprot.;Acidithiobacillales;Acidithiobacillaceae;Acidithiobacillus
172		1					1		1	Actinobacteria;Actinobacteria;Actinomycetales;Propionibacteriaceae;Propionibacterium
173									1	Actinobacteria;Actinobacteria;Coriobacteriales;Coriobacteriaceae
174								1		TM7;TM7_genera_incertae_sedis
175					1					Firmicutes;Bacilli;Bacillales;Alicyclobacillaceae;Alicyclobacillus
176	2		1							Proteobacteria;Gammaproteobacteria

CHAPTER 5: Enrichment and isolation of acidophilic sulfate-reducing bacteria from Tinto River sediments



INTRODUCTION

Sulfur is one of the most abundant elements on the Earth. It occurs in different oxidation states and chemical forms in the environment. Under aerobic conditions, sulfate is the thermodynamically stable form. Sulfate is formed by biological oxidation of sulfide in ores and sediments and anthropogenically by burning processes. Sulfate causes low direct environmental risk, but in the environment it can be reduced to sulfide (H_2S) by anaerobic microorganisms. H_2S is toxic, odorous and causes corrosion problems (27).

Sulfate reduction is mediated by a group of microorganisms, both bacteria and archaea, often termed sulfate-reducing bacteria (SRB), though the terms sulfate-reducing prokaryotes (SRP) and sulfate-reducing microorganisms (SRM) are also used. These microorganisms are characterized by their ability of dissimilatory sulfate reduction. This is a process by which the reduction of sulfate (SO_4^{2-}) to sulfide (S^{2-}) with organic electron donors or H_2 is coupled to energy conservation and growth. SRB play an important role in biocorrosion of concrete structures e.g. in wastewater systems (26) and ferrous metal installations such as pipes in the petroleum industry (5). Besides these unwanted properties of SRB, recent studies (15, 20, 34) address the bioremediation potential of SRB for the removal and recovery of sulfur, toxic heavy metals and radionuclides from waste streams. Generally, SRB prefer pH values between 6 and 8 for growth (37) but research has indicated that sulfate reduction at a pH<5 is possible (17). Thus far, only a few SRB that grow at low pH have been isolated (1, 28).

Previous studies in the extreme acidic environment of Tinto River (Huelva, Spain) indicated the presence and activity of a sulfate-reducing microbial community in Tinto River sediments with a pH of 3.8-5.4 (24, 25). The Tinto River (Huelva, Spain) is a natural acidic rock drainage (ARD) environment. The biological oxidation of metallic sulfides from the Iberian Pyritic Belt produces waters with low pH (pH 2.3) and high ferric iron (~2 g L^{-1}) and sulfate (~6 g L^{-1}) concentrations. Low pH and ferric iron facilitate metal solubilization (Cu: ~0.1 g L^{-1}; Zn: ~0.2 g L^{-1}, etc.) (14). Such metals might be immobilized or recovered by biological sulfate reduction. Using Tinto River sediments as inocula, a screening for sulfate-reduction was performed, studying the effect of different pH, inocula origins and electron donors, with the final aim to isolate SBR able to grow at low pH.

MATERIALS AND METHODS

Source of organisms: field site description and sampling

The acid sediments studied are located in Tinto River basin (Huelva), in southwestern Spain. Samples were collected from three sampling sites, *JL* dam (37.691207N, 6.560587W), *SN* dam (37.72173N, 6.557465W) and *SAL* (37.675125N, 6.551628W) in September 2011. At *JL* and *SN* dams, sediment cores (7 cm inner diameter and 45 cm length) were taken with a sampler (Eijkelkamp Agrisearch equipment, Giesbeek, The Netherlands). At *SAL*, the sample was taken from the accumulated sediment directly to a 50-mL tube. The redox potential (E) and pH of the drill core samples were measured *in situ* with E and pH probes, connected to a Thermo Orion 290A potentiometer placed into the fresh sediment just after extraction. *JL*, *SN* and *SAL* have a pH of 5.5, 3.2 and 2.3 and a redox potential of -118, 200 and 300 mV, respectively.

Media

(i) Liquid medium: A O_2-free, N_2-bubbled basal medium was prepared as described before (30) but bicarbonate-buffer was eliminated to allow pH modifications. The basal medium contained the following (g L^{-1}): 0.53 $Na_2HPO_4 \cdot 2H_20$, 0.41KH_2PO_4, 0.3 NH_4Cl, 0.11 $CaCl_2 \cdot 2H_2O$, 0.1 $MgCl_2 \cdot 6H_20$, 0.3 NaCl and 0.48 $Na_2S \cdot 9H_2O$; as well as the acid and alkaline trace elements (1 mL L^{-1} each) and vitamins (0.2 mL L^{-1}) also described. The basal medium was supplemented with 0.1 g L^{-1} BBL yeast extract (Becton Dickinson, Cockeysville, Md.) and 1.42 g L^{-1} Na_2SO_4. The pH was adjusted before autoclaving according to the final desired pH. All compounds were heat-sterilized except for the vitamins and the solution of Na_2S, which were filter-sterilized. Organic substrates (glycerol, lactate, methanol and succinate) were added from 1 M anaerobic sterile stock solutions to give final concentrations of 5 mM. Routinely, cultures were incubated statically at 30°C in 120-mL serum vials with 50 mL of medium, sealed with butyl rubber stoppers (Rubber BV, Hilversum) and a gas phase of 1.5 atm N_2/CO_2 (80:20, v/v). For the cultivation of hydrogen-consumers, H_2/CO_2 (80:20, v/v) was used and 2 mM of acetate was supplemented as carbon source as recommended elsewhere (37). **(ii) Solid media:** To prepare agar plates, agar (0.8% agar noble; Difco) was added to the medium described above. Slight modifications to distinguish SRB from other bacteria were performed: Mohr´s Salt (0.1%) was added and L-cysteine (0.05%) was used as reducing solution instead of Na_2S. Plates were prepared in an anaerobic chamber and left to dry overnight.

Experimental set-up

Screening: A screening for sulfate reduction was performed with Tinto river sediments making variations in medium conditions. Five mL of sediment samples from three different origins (*JL*, *SN* and *SAL*) were diluted in basal media (1:10). The range of pH tested was from 4 till 6.5 with 0.5 increments. Five different electron donors were used: succinate, glycerol, methanol (just for *JL*), lactate and hydrogen. In this set of 78 bottles the sulfate-reducing activity was followed by measuring pH, sulfate conversion to sulfide and organic compound transformations. According to the results, ten enrichments were selected for molecular characterization of the 16S rRNA gene sequence and for further enrichment and isolation of SRB. Selected enrichments from the screening were periodically transferred to fresh medium with the same pH and electron donors.

Isolation: Taking into account some difficulties to grow SRB on plates at low pH, the serial dilutions approach was first applied using a 10% inoculum size into fresh media. The highest dilution that showed growth after 3-4 weeks of incubation was used for further isolation. Serial dilution was performed three times. Finally, pure cultures were obtained by plating. Aliquots of 0.1 mL of diluted samples were spread on agar plates in an anaerobic chamber. The streaking procedure was repeated three times to obtain pure cultures. Plates were incubated upside down in a gas tight jar with the anaerobic chamber atmosphere (96:4 N_2/H_2) and containing an O_2 scavenger (Anaerocult mini, Merck) at 30°C. Finally single colonies were picked up and inoculated into serum bottles. To check purity, isolates were inoculated into the corresponding medium with 1 g L^{-1} BBL yeast extract (Becton Dickinson, Cockeysville, Md.). After incubation, the cultures were examined microscopically for purity and the 16S rRNA genes of the pure cultures were sequenced.

Clone library construction for 16S rRNA gene phylogenetic analysis.

Total DNA was extracted from 10 mL of each culture using FastDNA® SPIN® Kit (for Soil) (Qbiogene, USA) according to the manufacturer´s instructions. The 16S rRNA genes were amplified with the primers set 27F-1492R (T_a = 57°C) for Bacteria with GoTaq (Promega, Madison, WI) according to the manufacturer´s instructions. PCR products were purified with DNA Clean and Concentrator 25™ from Zymo Research and cloned in *Escherichia coli* DH5α competent cells by using the pGEM-T vector (Promega, Madison, WI) according to the manufacturer's instructions. Recombinant clones were identified as white colonies on chromogenic indicator plates. Isolated clones were transferred to 100 µL MilliQ water and thermic lyses was performed at 94°C for 10 min. Eluate (2 µL per 50 µL final volume) was used as template in a plasmid DNA PCR using SP6/T7 primers (Ta = 45°C). Plasmid DNA of positive clones was screened by Amplified Ribosomal DNA Restriction Analysis (ARDRA) using endonuclease BfuCI (1 U, 4 h, 37°C) and grouped according to the restriction patterns obtained. Two members of each group were then sequenced. Sequences were assembled using DNABaser programme and prior to phylogenetic analysis, vector sequences flanking the 16S rRNA gene inserts were identified using VecScreen tool (NCBI) (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.htmL). Clone sequences were checked for chimera using Bellerophon tool (13) and compared with the NCBI databases using the Ribosomal Database Project (4) to identify the closest sequence. The 16SrRNA genes sequences determined in this study have been deposited in the GenBank database under accession numbers - - for the pure and highly purified cultures.

Other methods

Sulfate was measured using a Dionex 1000 ion chromatograph. Organic compounds were quantified with an LKB high-performance liquid chromatograph (HPLC) as described previously (30). Sulfide was measured photometrically with methylene blue method (3). Phase contrast photographs were made with a Leica DM2000 microscope. Gram staining was done according to standard procedures (6). Statistic analysis was performed with FastUniFrac (10).

RESULTS

Screening

Sulfate reduction hardly occurred in the enrichments with the *SAL* sample where mainly fermentative growth was observed. Enrichments from this sample were not further analyzed. For an easier comprehension, the data set of sulfate and organic acids measurements in *JL* and *SN* enrichments is summarized in figure 1, the results of each enrichment are shown in a semi-quantitative way with the origin of the inoculum, the electron donor and pH of incubation.

Sulfate reduction occurred over a wide range of pH; a depletion of almost 4 mM of sulfate had occurred at day 15 in most enrichments in the whole range of pH (from 4 till 6.5). The sulfate reduction rates (SRR) of the enrichments averaged (mM day^{-1}): 0.12 at pH 4, 0.15 at pH 4.5, 0.16 at pH 5, 0.15 at pH 5.5, 0.14 at pH 6 and 0.1 at pH 6.5. Higher sulfate reduction rates were found at pH 5 than at circumneutral pH, suggesting an acidophilic nature of the microbiota inhabiting the enrichments.

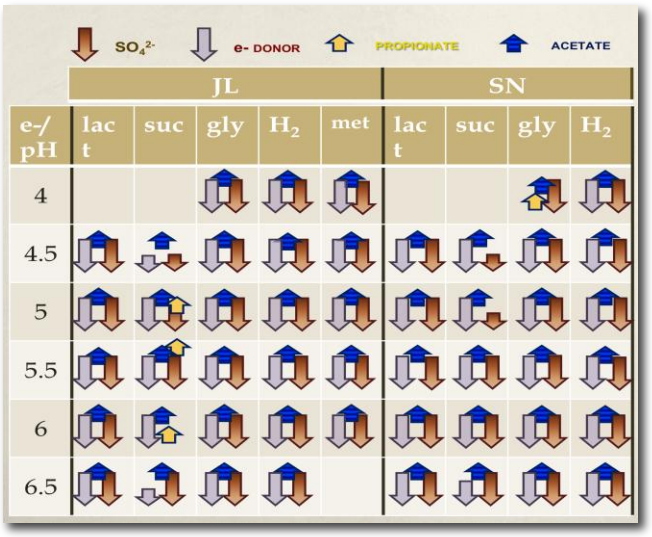


Fig. 1: Screening scheme of the enrichments reflecting the origin of the sediments (*JL* and *SN* sampling sites) incubated at pH 4 till pH 6.5 with succinate, glycerol, methanol, lactate or hydrogen as electron donors. Arrows summarize sulfate depletion and organic acids variations.

Measurement of pH resulted to be a good indicator of the occurrence of sulfate-reduction. In the bottles where sulfate-reduction had occurred, an increase in pH was observed; depending on the initial pH the increment could be up to 2 pH units. Positive results with respect to sulfate reduction were obtained till pH 4 with glycerol, methanol and hydrogen as electron donors, till pH 4.5 with lactate and till pH 5 with succinate. Acetate was detected in all the enrichments where growth was observed. In *JL* enrichments with succinate at pH 5, 5.5 and 6, propionate appeared to be an important product.

Selected enrichment cultures

Based on the observations, 10 enrichments were pre-selected, two per electron donor prioritizing those with lower pH values (Fig. 2).

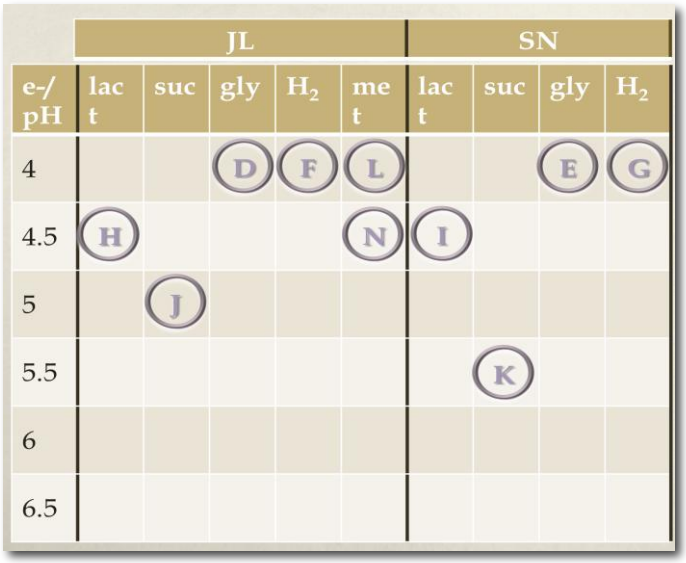


Fig. 2: Screening scheme of the enrichments reflecting the origin of the sediments (JL and SN sampling sites) incubated at pH 4 till pH 6.5 with succinate, glycerol, methanol, lactate or hydrogen as electron donors. Circles show the selected bottles for further purification.

Serial dilution method was applied with material of these 10 bottles in order to get axenic cultures of acidophilic SRB. Physicochemical changes were also monitored. In just 5 days sulfate-reducing activity was observed with a depletion of sulfate added ranging from 3% till 36% and an increment of pH from 0.3 till 1.1 pH units. Acetate formation was observed in all growing cultures. Remarkably, culture J and N transferred enrichments showed mainly fermentative activity with propionate production.

Microbial populations were analyzed by cloning and sequencing to know the taxonomic position of the responsible microbes. The detected bacteria fall into 4 different phyla: *Actinobacteria* within *Actinobacteria* class (2%), *Bacteroidetes* within *Bacteroidetes* class (11.4%), *Proteobacteria* within *Beta* and *Gamma* classes (9.9%) and *Firmicutes* within *Clostridia* class (76.6%). In total, 11 genera were enriched: *Paludibacter, Variovorax, Pseudomonas, Thermodesulfobium, Propionispora, Cellulomonas, Clostridium, Oscillibacter, Desulfitobacterium, Desulfosporosinus* and *Desulfotomaculum*. Remarkably, presumed fermentative bacteria and SRB were co-enriched. Statistic analysis via Fast UniFrac showed that from the three parameters studied - pH, electron donors and source -, just the latter affected the microbial distribution in our enrichments (Fig. 3).

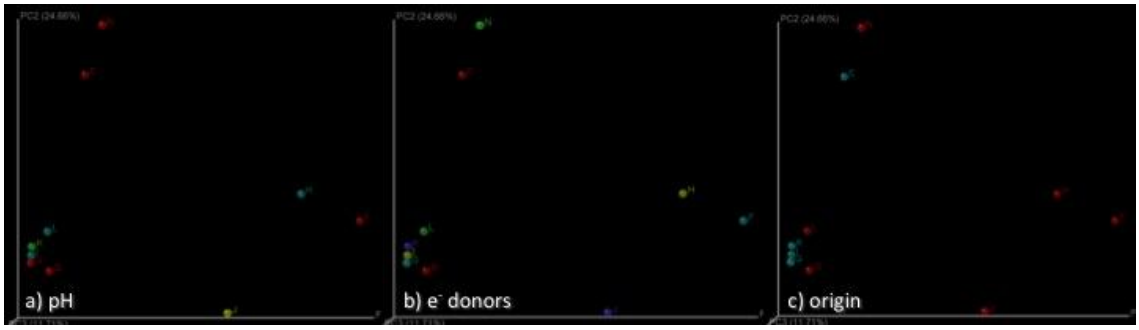


Fig. 3: Fast Unifrac analysis of the ten cultures with different factors: a) pH, b) electron donor and c) origin.

The distribution of the different species and their morphology in the different conditions are summarized in figure 4.

Isolation

After repeated transfers and four serial dilutions in fresh media, the cultures were highly purified but in this stage not yet isolated. Together with the most predominant bacteria, small rod-shaped bacteria remained present in low numbers (less than 1%) in all the cultures, likely due to the presence of yeast extract. By adding extra yeast extract to highly enriched cultures, this contaminant increased in number, suggesting a fermentative metabolism. By passing the culture L over a 0.45 µm filter, SRB were retained while the smaller fermentative contaminant passed the filter. The contaminant could be enriched and isolated with yeast extract as substrate, which is fermented to acetate, and propionate in a molar ratio of about 5:3:1. This bacterium was also able to grow with glucose but not with pyruvate or lactate. The phylogenetic analysis showed a similarity of only 92% with the closest cultures isolate, which is *Paludibacter propionicigenes* WB4 (CP002345).

For further isolation of SRB, four enrichments (D, E, L and I) were selected because of their lower similarity to cultured relatives. The chemical analysis of the enrichments showed a total depletion of the added electron donors, reduction of sulfate and production of acetate.

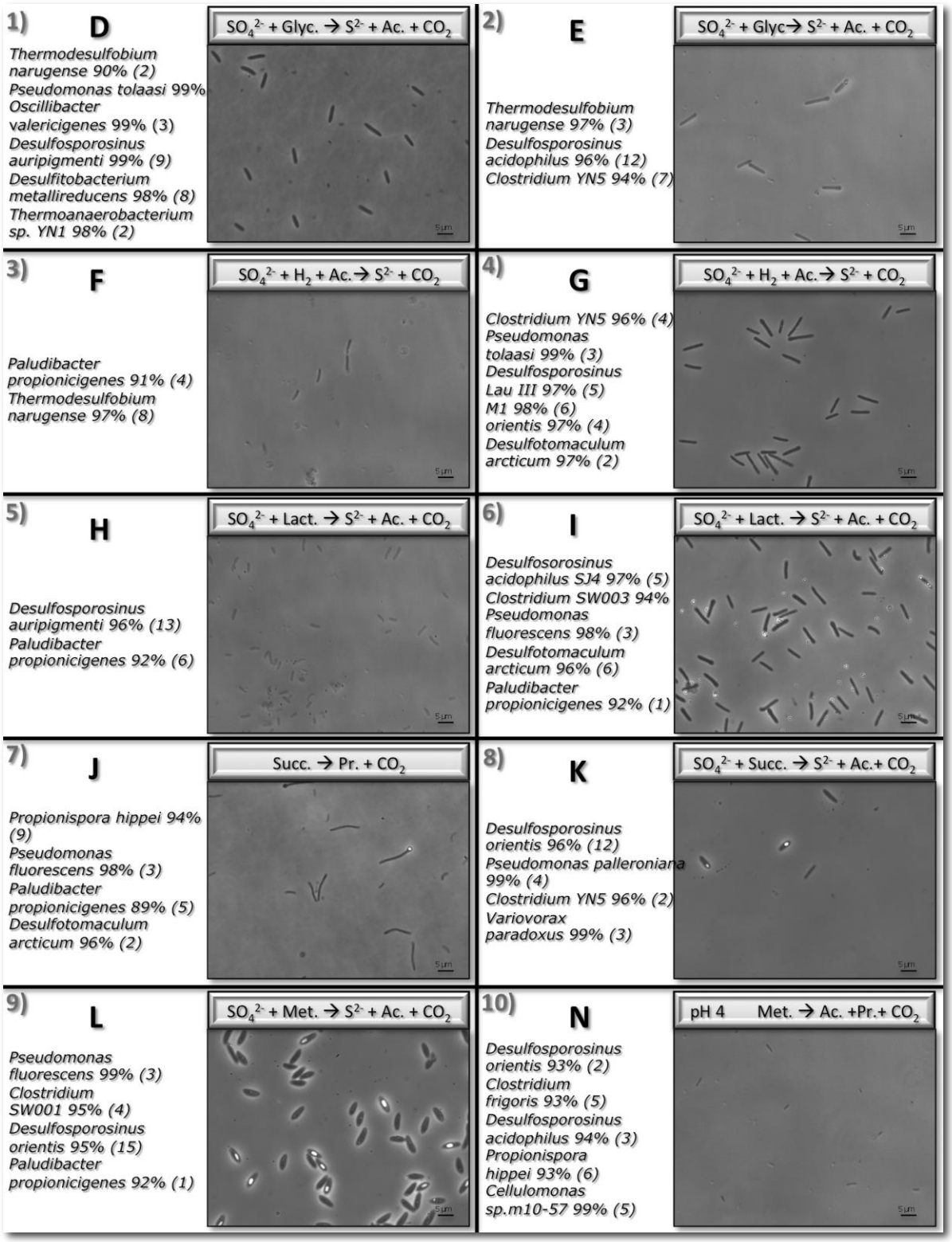


Fig. 4: Scheme of the ten selected cultures: phylogenetic affiliation and number of clones, morphology and measured transformations taking place in the bottles.

The enrichments reduced sulfate and oxidized the added electron donors (glycerol, lactate, and methanol) as indicated in Table 1.

Table 1: Stoichiometry of sulfate and electron donor utilization by the enrichments.

	Electron acceptor Consumption		Electron donor consumption		Acetate production	
Enrichment	**mM**	**meq redox**	**mM**	**meq redox**	**mM**	**meq redox**
D (glycerol)	3.3	26.4	5.2	31.2	5.3	31.8
E (glycerol)	3.5	28	4.6	27.6	5.4	32.4
I (lactate)	2.7	21.6	5.4	21.6	6.1	24.4
L (methanol)	3.6	28.8	5.5	30.6*	0.6	2.4

***From 1 mol methanol, 0.15 is converted till acetate via reaction 5 and 0.85 is oxidized via sulfate reduction (reaction 1 and 4).**

According to the equations shown in Table 2, 1 mol glycerol and lactate will be partially oxidized to 1 mol acetate with the concomitant reduction of 0.75 and 0.5 mol sulfate, respectively. In both cases, higher acetate production than theoretically expected was observed. It can be due to an acetate formation via acetogenesis (eq. 6, Table 2) from CO_2 and the H^+ liberate from electron donor oxidation. Methanol metabolism can follow two ways. On the one hand, 1 mol methanol can be totally oxidized till carbon dioxide reducing 0.75 mol of sulfate (reaction 4) and on the other hand, 1 mol of methanol can be partially oxidized till 0.5 mol acetate with the reduction of 0.25 mol of sulfate (reaction 5). The ratio between both reactions will determine the final stoichiometry.

Table 2: Metabolic reactions in the cultures.

(1) Sulfate	$SO_4^{2-} + 8H^+ + 8e^- \rightarrow S^{2-} + 4H_2O$
(2) Glycerol	$C_3H_8O_3 + H_2O \rightarrow C_2H_4O_2 + CO_2 + 6H^+ + 6e^-$
(3) Lactate	$C_3H_6O_3 + H_2O \rightarrow C_2H_4O_2 + CO_2 + 4H^+ + 4e^-$
(4) Methanol A	$CH_4O + H_2O \rightarrow CO_2 + 6H^+ + 6e^-$
(5) Methanol B	$4CH_4O + 2CO_2 \rightarrow 3C_2H_4O_2 + 2H_2O$
(6) Acetogenesis	$2CO_2 + 8H^+ + 8e^- \rightarrow C_2H_4O_2 + 2H_2O$

To ensure separation of the enrichments from the fermenting bacteria, inoculation on agar plates was done. The D, E and L cultures grew on agar plates, but culture I did not. The SRB in culture I could be isolated by performing another 2 extra dilution series. As common features, the sulfate-reducing isolates consisted of gram-positive, rod-shaped and spore-forming bacteria with ellipsoidal spores causing swelling of the cells, but differ in sizes or spore-position. From culture D rod-shaped bacteria of 1.1 by 3-5 µm with subterminal spores were isolated, cells from culture E were 0.7 by 2 µm with subterminal-spores, bacteria in culture I were straight rods 0.7 by 4 µm with terminal spores and culture L were 1.1 by 2-3 µm and had central spores.

Sequencing of the 16S rRNA genes was performed with these 4 cultures. The phylogenetic tree based on 16S rRNA genes is shown in figure 5.

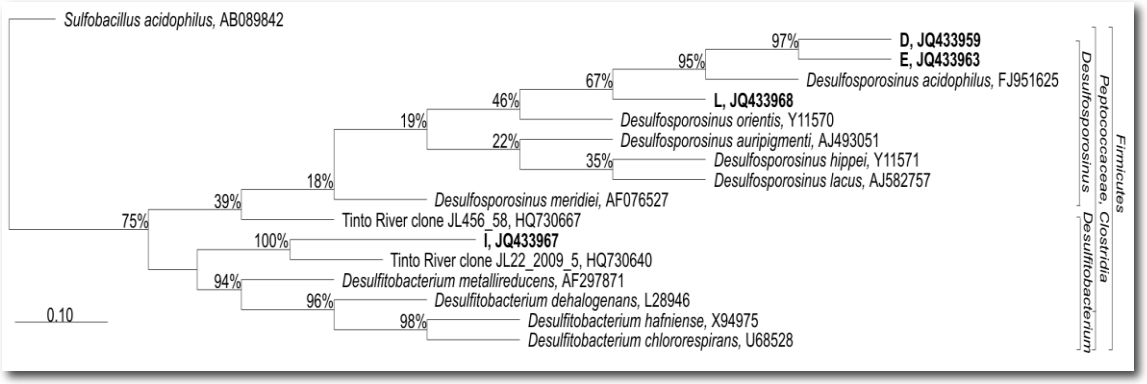


Fig. 5: Phylogenetic affiliations of 16S rRNA sequences of the four isolates. The consensus phylogenetic tree was generated using parsimony, neighbour-joining, and maximum likelihood analyses with different sets of filters, which showed stable branching. One hundred bootstrap replicates were performed. The bar indicates a 10% estimated sequence divergence. The sequences from the isolates are indicated in boldface type. Designations after the organism names or identifiers are GenBank accession numbers.

Culture I clustered into a group without close cultured representatives. The closest sequences belong to clone HQ730640, retrieved by cloning in a former study of Tinto River sediments (25). The closest but distantly related cultured representatives are *Desulfitobacterium metallireducens* strain 853-15 (93%) and *Desulfosporosinus orientis* DSM 765 (94%). Culture L sequence have 96% similarity with *Desulfosporosinus orientis* DSM 765 (NR_026411) and *Desulfosporosinus acidophilus* SJ4 (FJ951625). Culture D and E had 96% similarity with *Desulfosporosinus orientis* DSM 765 (NR_026411) and 96% and 97%, respectively with *Desulfosporosinus acidophilus* SJ4 (FJ951625), suggesting that the D and E cultures are phylogenetically similar (2.2% of sequence differences). A clone sequence (HQ730667) related to these three isolates was found in a clone library from Tinto River sediments (25).

DISCUSSION

At low pH bacteria need to invest energy to maintain a high internal pH, which makes bacteria with a low metabolic energy yield, such as SRB, sensitive to low pH (11). As a consequence, SRB are considered to prefer an environment with a pH between 6 and 8. But, in this study, sulfate reduction took place over a wide range of pH (tested from 4 till 6.5). Interestingly, they can grow at low pH, but increase the pH till almost 7. The lowest pH allowing growth strongly depended on the electron supplied, even at the low concentration added (5 mM). Organic acids are inhibitory at low pH as they may pass the cell membrane in the undissociated/acid form acting as an uncoupler (9). Their toxicity depends on their dissociation constants, the pK_a of lactic acid is 3.08 (K_a=8.3x10^{-4}) meanwhile pK_1 of succinate is 4.21 (Ka=6.16x10^{-5}) and pK_2 is 5.64 (K_a=2.2x10^{-6}). This is in line with our results showing that lactate inhibition at concentration 5 mM starts below 4.5 and succinate below 5.5. On the other hand, the non-ionic substrates (glycerol, H_2 and methanol) allowed sulfate reduction at pH 4 and likely even lower. By using the followed approach of the serial dilution to extinction method, low electron donor concentration and preferably non-ionic substrates, it is possible to enrich acidophilic SRB.

From the fermenters found, the bacterium related with *Paludibacter propionicigenes* was the more resistant after the transfers. *P. propionicigenes* is a strictly anaerobic, gram-negative,

propionate-producing bacterium (33), what may explain the propionate production detected in several cultures. Related sequences have been found before in Tinto River by DGGE and cloning of environmental DNA (25) and others acidic environments (DQ205192) suggesting that such types of bacteria can adapt well to acidic conditions. Currently, the physiological properties of this bacterium are studied further.

Thus far, just a few acidophilic/tolerant SRB have been isolated or obtained in stable enrichment cultures (1, 2, 12, 16, 18, 28, 32). In this study, 4 novel acidophilic SRB strains were isolated. For three isolated SRB the highest similarity with characterized species is only 96%; two of them to *Desulfosporosinus acidophilus* and one to *Desulfosporosinus orientis*. The fourth one has just 93% 16S rRNA gene sequence similarity with the clade *Desulfitobacterium/Desulfosporosinus.* The first three strains could be new *Desulfosporosinus* species, while the fourth one could be a new *Desulfitobacterium* species or according to the 16S rRNA sequence, even a new genus.

Members of the genera *Desulfitobacterium* and *Desulfosporosinus* represent a clade of strictly anaerobic, rod-shaped, and sporeforming bacteria, affiliated with the family *Peptococcaceae*, order *Clostridiales,* phylum *Firmicutes.* Representatives of the genus *Desulfosporosinus* can be easily distinguished by their ability to reduce sulfate, autotrophically with H_2 and heterotrophically with lactate. By contrast, the key feature of members of the genus *Desulfitobacterium* is their ability to use a wide spectrum of electron acceptors, including chlorinated aliphatic and aromatic compounds for reductive dehalogenation. Strains of both genera are able to utilize sulfite and heavy metals as electron acceptors (29). In permanently anaerobic environments, sporeforming species are apparently less competitive than non sporeforming ones (38) but in contrast, sporeforming are able to survive dryness and oxic conditions for months what make them dominant in adverse conditions. *Desulfosporosinus* members have previously been detected in other acidic environments, including acidic mining-impacted lake sediments (18) and Tinto River (7, 25) where *Desulfitobacterium* was also found (25).

Desulfosporosinus genus belongs to the SRB group that degrade organic compound incompletely to acetate (1, 28), indeed, acetate accumulation was observed in the enrichments. *Desulfosporosinus* spp. are known to switch under sulfate limitation to the fermentation of lactate and pyruvate (29), to reductive acetogenesis from formate, methanol or methyl groups of aromatic compounds (22) or to dissimilatory iron (III) reduction (23). This bacterium was proposed to be important for the carbon flow in terrestrial ecosystems (19, 21) and on globally relevant processes such as the decrease in emission of the greenhouse gas methane (8). There is some evidence that syntrophic relationships occur between *Desulfitobacterium* spp. and sulfate-reducing bacteria such as *Desulfosporosinus* spp., from which the *Desulfitobacterium* cells acquire their electrons by interspecies hydrogen transfer. Their versatility makes desulfitobacteria excellent candidates for the development of anaerobic bioremediation processes. In fact, desulfitobacteria have been detected in bioprocesses treating sites contaminated with halogenated organic compounds but also with high concentrations of toxic metals (35).

Acidotrophic SRB may become important for the bioremediation of acid mine drainage (AMD) waters, which are highly acidic (pH<4) and have a high dissolved metals content. The dissimilative reduction of sulfate leads to a consumption of protons which increase the pH, and

to sulfide formation which reacts with heavy metals such as Fe, Zn, Cu, Cd, Ni, and Pb, leading to the precipitation of insoluble metal sulfide phases and consequently, removal of metals from solution (15). Additionally, some metals can be enzymatically reduced such as Cr(VI), Mn(IV), Fe(III) or U(VI) (31). Remarkably, dissolved uranium, U (VI), may be removed by reduction to U(IV) creating the insoluble mineral uraninite (36). Further studies are needed to get insight into sulfate reduction and metal reduction by acidophilic SRB.

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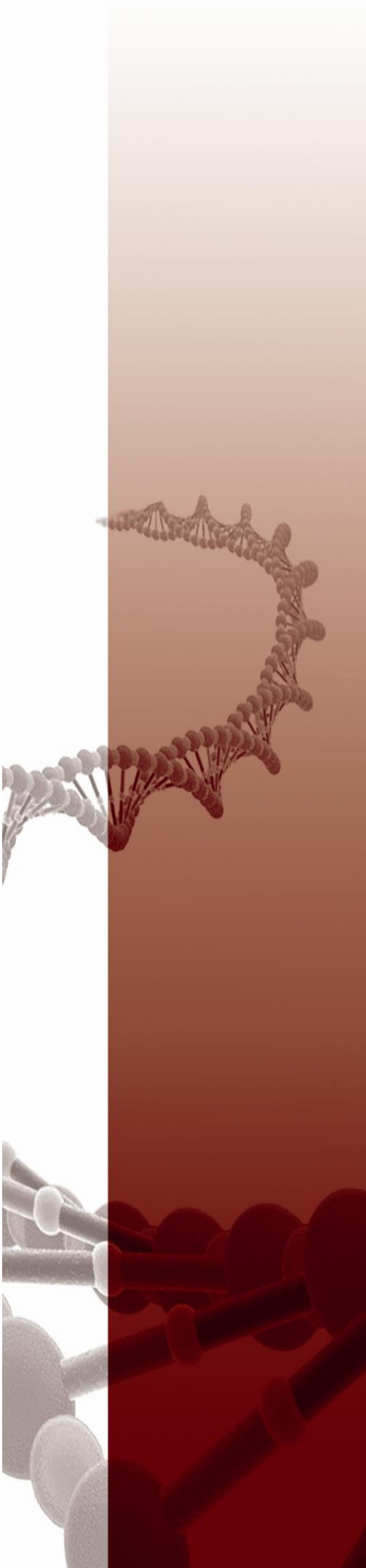
CHAPTER 6: Bioremediation of acid mine drainage coupled with domestic wastewater treatment



INTRODUCTION

Acid mine drainage (AMD) is characterized by a low pH and high concentrations of sulfate and heavy metals. Although it occurs naturally, it is mainly associated with mining activities. When a sulfide-bearing material is exposed to oxygen and water, iron- and sulfur- oxidizing chemolitoautotrophs, such as *Acidithiobacillus ferrooxidans,* can accelerate AMD production by assisting in the breakdown of sulfide minerals. Mining activities and deficient mine waste management cause the formation and release of AMD into the environment, reaching waterways and groundwater, producing long-term harm to water quality and biodiversity. Furthermore, some effluents generated by the metal mining industry contain large quantities of toxic substances, such as cyanides and heavy metals, which have serious human health and ecological implications. Globally, AMD has become a major environmental concern because of its toxicity, extent and worldwide distribution (17).

Several physicochemical methods have been developed for the treatment of AMD. Traditionally, metal containing wastewaters have been neutralized with alkalis, such as lime or calcium carbonate, since, in their presence, heavy metals precipitate in form of hydroxides or carbonates. This method, however, has some disadvantages such as its operational and maintenance costs, and the production of large quantities of sludge (4). Biological treatment of AMD with sulfate-reducing bacteria (SRB) has been under research as a promising alternative to chemical methods. Sulfate-reducing metabolisms are widespread among different anaerobic *Bacteria* and some *Archaea*, however, literature usually uses the term SRB to include them all (16). SRB are able to degrade a large amount of different substrates to reduce sulfate to hydrogen sulfide (18), preferring simple organic compounds or hydrogen as electron donors. The hydrogen sulfide produced reacts with dissolved metal ions forming low solubility metal sulfide precipitates. The SRB concurrently consume hydrogen ions and produce carbon dioxide which generates alkalinity, thereby increasing pH levels (11).

Application of this biological feature to AMD remediation is usually carried out in bioreactors because important variables such as pH, hydraulic retention time (HRT), sludge retention time (SRT) and organic loading, can be better controlled and optimized for long term performance. In active sulfate reduction bioreactors, the main variable when evaluating the cost effectiveness of this method is the electron donor used. Several simple compounds (e.g. lactate, ethanol) (11, 13) and complex substrates (e.g. molasses, manure) (3) have been used. In this study domestic wastewater (DW) is used as source of organic matter and macronutrients (nitrogen and phosphorous) for SRB. The advantages of this process, when compared to conventional neutralization/precipitation methods, are: (i) cost-effectiveness, as it uses a waste stream; (ii) lower metal concentrations in the effluent; (iii) metal sulfide complexes are less voluminous and chemically more stable when they are stored under anaerobic conditions (14) and finally, (iv) valuable metals can be recovered from the metal sulfide sludge.

Microbial communities are responsible for the process, so a good election of the inoculum is crucial. Previous studies in the extreme acidic environment of Tinto River (Huelva, Spain) have suggested the presence and activity of a sulfate-reducing microbial community in its anaerobic zones (20, 21), therefore Tinto River sediments were used as inocula. During the operation, the study of the microbial communities inhabiting bioreactors is crucial in understanding its performance, e.g. when a complex source of nutrients is used in the process (such as DW), the

efficiency depends on a consortium rather than SRB alone (14). Another key factor in sulfate-reducing bioreactors is the microbial support to avoid biomass loss (17).

The aim of this study was to assess the feasibility of coupling AMD bioremediation with the treatment of DW through a sulfate reduction process in anaerobic bioreactors. Three different bacterial supports were evaluated by following the efficiency of the process (sulfate reduction, metal precipitation and pH attenuation) and examined through SEM. Influence of HRT, influent pH and extra addition of electron donors were also tested. Bacterial diversity and shifts were examined at different times during the study through Denaturing Gradient Gel Electrophoresis (DGGE) analysis.

MATERIALS AND METHODS

Site sampling and inocula preparation

Sediments from the Tinto River basin (Huelva, southwestern Spain) were used as inoculum. Samples were collected from the JL dam (37.691207N, 6.560587W) in November 2010. Sediment cores (inner diameter, 7 cm; length, 45 cm) were taken with a sampler (Eijkelkamp Agrisearch Equipment, The Netherlands). The redox potential (E) and pH of the drill core samples were measured *in situ* with E and pH probes connected to a Thermo Orion 290A potentiometer placed in the fresh sediment just after extraction of the core. The cores were sliced according to both physicochemical parameters and visual aspect; blackish slices (pH between 4.5 and 5.5 and redox potential between 0 and -130) were kept separate until processing in the laboratory (less than 1 week).

An enrichment culture was grown in 1 L- anaerobic bottle containing 100 g of sediment and 500 mL of the following sterilized medium (in g L^{-1}): 3.5 sodium lactate, 2 yeast extract, 2 $MgSO_4 \cdot 7H_2O$, 1.5 Na_2SO_4, 0.5 $K_2HPO_4 \cdot 3H_2O$, 0.5 L-Cysteine, 0.4 $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.1 $CaCl_2$, plus 1.0 mL of a mixture of trace elements; pH was adjusted to 5.5. The trace element solution was prepared as follows (in mg L^{-1}): 500 EDTA, 39.0 $ZnSO_4 \cdot 7H_2O$, 50.6 $MnCl_2 \cdot 4H_2O$, 11.0 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 15.7 $CuSO_4 \cdot 5H_2O$, 16.1 $CoCl_2 \cdot 6\ H_2O$. The culture was incubated statically at 30 °C. After one month of incubation, an anaerobic bottle of 1 L containing 500 mL of medium and 150 g of granular activated carbon (Chemviron F-400, Aguas de Levante, Spain) was inoculated with 10% inoculum from the aforementioned enrichment.

Bacterial supports

SRB supports for the three reactors were prepared as follows. For reactor 1 (R1) granular activated carbon (AC) from the second enriched culture described above was placed in the reactor. For reactor 2 (R2), sediment from the first enriched culture described was mixed in a 1:1 proportion with melted agar (1% at 41 °C). The gel formed was cut with a sieve of a 2 mm diameter. For reactor 3, (R3) 500 ml of the supernatant from the second enrichment culture was concentrated to 100 ml through centrifugation, and mixed with 100 ml of melted agar (2% and 41°C). The gel obtained was cut with a sieve of a 2 mm diameter. The bacterial supports were incubated in batch mode for 3 weeks in the AMD-DW medium, which was replaced weekly.

Experimental design

Synthetic AMD was made with a composition close to that observed in Tinto River (7). Two solutions were prepared with the same metal concentration but different iron species (Fe^{2+} and Fe^{3+}) (in mg L^{-1}): 11449 $FeSO_4 \cdot 7H_2O$ or 8522 $Fe_2(SO_4)_3 \cdot H_2O$, 660 $ZnSO_4 \cdot 7H_2O$, 196 $Mn(NO_3)_2 \cdot 4H_2O$, 196 $CuSO_4 \cdot 5H_2O$, 40 $CoCl_2 \cdot 6H_2O$, 9 $NiSO_4 \cdot 6H_2O$. DW was collected from the campus wastewater treatment plant of the Autonomous University of Madrid, which had a mean dissolved COD of 400 mg-COD L^{-1}. The theoretical sulfate reducing ratio between COD and sulfate is established at 0.67 mg-O_2/mg-SO_4^{2-} (8).

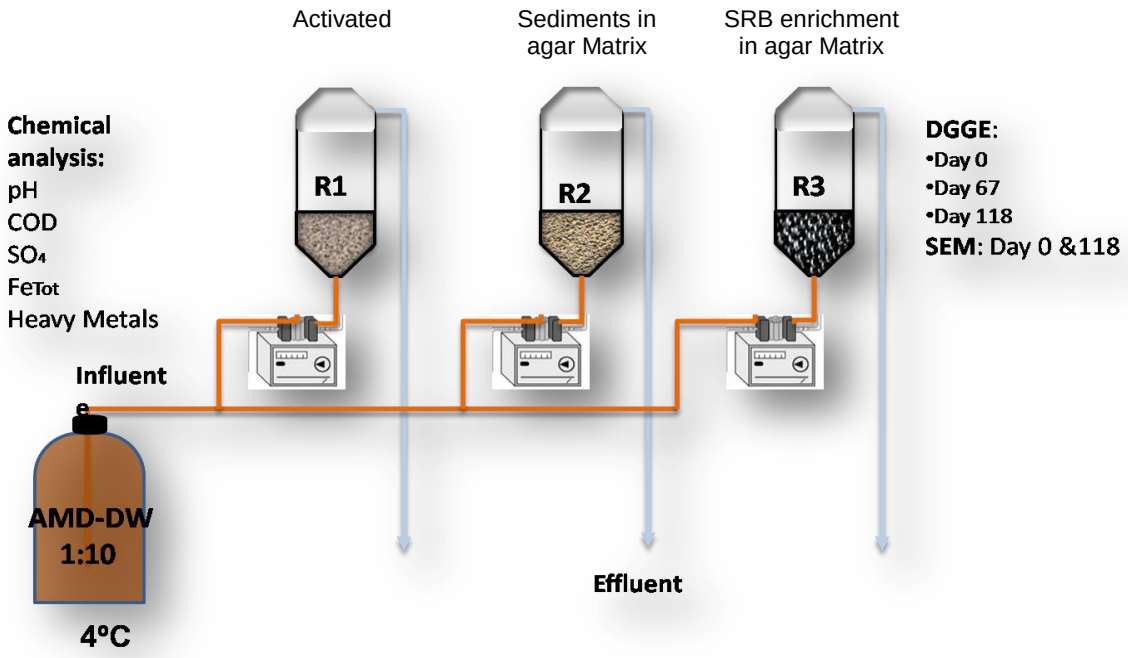


Fig. 1: Scheme of the experiment. Three laboratory-scale upflow anaerobic sludge bed (UASB) reactors (0.5 l) were operated for 118 days at 30 ± 1°c fed with a 1:10 mixture of acid mine drainage (AMD) synthetic water and domestic water (DW).

Three laboratory-scale Upflow Anaerobic Sludge Bed (UASB) reactors (0.5 L) were operated for 118 days at 30 ± 1°C (Fig.1). The reactors were fed with a 1:10 ratio of AMD:DW, experimentally implemented, with the addition of acetate (150 mg L^{-1}) from day 71 on. The AMD:DW mix was prepared twice a week (stored at 4°C) and precipitates were removed from the feed tank once a week. The experiment was divided into five phases. Variable settings are shown in Table 1. Phase I corresponds to the adaptation of the inocula and stabilization of the system. In phase II the HRT was fixed at 48 h. From phase III onwards, the influent pH was adjusted to 5 with the addition of HCl or NaOH when necessary. In phase IV the addition of acetate began and finally in phase V the HRT was reduced to 24 h. COD, sulfate, total Fe and pH were measured twice a week during phases I to IV and three times during phase V. Metals were measured twice for every operational phase. Rates of sulfate reduction, dissolved COD removal and total Fe precipitation were determined as the difference between feed and effluent concentrations.

Table 1: Bioreactor continuous operational phases

Phase	Time (d)	HRT (h)	pH influent	Influent	Biological Tracking
I	0-11	24-48	5.5-6	AMD:DW 1:10	SEM+DGGE day 0
II	11-29	48	4.8-6.3	AMD:DW 1:10	-
III	29-71	48	5	AMD:DW 1:10	DGGE day 67
IV	71-99	48	5	AMD:DW 1:10+Ac	-
V	99-118	24	5	AMD:DW 1:10+Ac	SEM+DGGE d 118

Chemical analyses

Samples were centrifuged at 13000 x g for 10 min and the supernatant taken for analysis of COD, sulfate and dissolved total Fe. The COD was determined according to method 5220D, Standard Methods APHA (9) using a COD Reactor (Hach, USA) and a spectrophotometer (Pharma Biotech Novaspec II, Sweden). Sulfate concentration was determined by ion chromatography with a suppressed conductivity detector (790 Personal IC with an A Supp 5 250/4.0 column Omega Metrohom, Switzerland). Measurements for pH were obtained with an Orion 2-Star portable pH meter (USA). Iron species (ferric, ferrous and total) were determined by reflectrometry (Iron test method 1.16983.0001, Reflectoquant, Merck, Germany). For the determination of dissolved metals, samples were stabilized with nitric acid and were determined by inductively coupled plasma (ICP-MS Elan 6000 Perkin Elmer Sciex, USA).

Denaturing Gradient Gel Electrophoresis (DGGE)

Microbial samples for 16S rRNA analyses were taken at different times throughout the experiment: at the starting point (day 0) from the sediment and the enrichment culture before support preparation (AC), at day 67 (before acetate supplementation) and day 118 (end of the experiment) from the three reactors (R1, R2 and R3). Total DNA was extracted from 10 g of each sample using FastDNA Spin kit for soil (Qbiogene, Inc., USA). Adaptation of the commercial protocol was carried out to optimize it for samples with high concentrations of heavy metals and a low biomass content as described previously (20). First, the complete 16S rRNA gene was amplified with primer sets 27F-1492R. The PCR conditions were as follows: 10 min of initial denaturation at 94°C and 35 cycles at 94°C for 1 min and annealing at 56°C for 1 min and 72°C for 2 min, followed by 10 min of final primer extension. The V3 to V5 variable regions of the 16S rRNA gene were amplified with the primer set 341F (GC)-907R. Some PCR parameters adapted were: 30 cycles, annealing temperature [T^a=52°C] and elongation time [1 min]. Primers 341F (GC) included a GC clamp: 5-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCC CCG CCC-3. The amplification reaction was performed according to the Taq DNA polymerase protocol (Promega, USA). An Applied Biosystem thermal cycler (USA) was used. DGGE analysis was carried out using a D-Code universal detection system instrument (Bio-Rad, USA). Polyacrylamide (6%; 37.5:1 acrylamide-bisacrylamide) gels with a 40 to 70% urea-formamide (100% urea-formamide contains 7 M urea and 40% deionized formamide) denaturant gradient were used in TAE (Tris-acetate-EDTA) buffer, pH 7.4, at 200 V for 4 h at 60°C. Gels were stained with ethidium bromide and visualized under UV illumination. About 60 bands were cut from the gel with sterile blades and placed in sterile vials with 50 µl of Milli-Q water.

DNA was allowed to diffuse into the water at 4 °C overnight. Five microliters of the eluate was used as a DNA template in a PCR mixture of 50 µl with the primers described above but without the GC clamp. A total of 57 bands were successfully sequenced using a Big-Dye

sequencing kit (Applied Biosystems) following the manufacturer's instructions. Sequencing chromatograms were checked with the program Finch TV v.1.4.0 (Geospiza). A comparative analysis of all the sequences was done using the BLAST routine from NCBI employing the GenBank database (http://www.ncbi.nlm.nih.gov/Blast) and the tool Sequence Match from the Ribosomal Database Project (RDP) at Michigan State University (http://rdp.cme.msu.edu/). Sequences were deposited in GenBank under accession numbers: JQ517512-JQ517538.

Scanning electron microscopy (SEM)

Samples were taken at the starting point (day 0) and at the end of the experiment (day 118) and fixed by immersion in glutaraldehyde (2.5%) for 2 h, then washed twice in sodium cacodylate buffer (0.2 M, pH 7.1). Samples were dehydrated in a graded series (10, 30, 50, 70, 90, and 100%) of ethanol/water mixtures, leaving them 20 min in each mixture. After dehydration, samples were critical point dried and mounted on stubs. After gold shadowing, samples were examined in a Phillips XL30 EDAX DX4i SEM (Netherlands).

RESULTS AND DISCUSSION

Influent design

The iron species (Fe^{2+} or Fe^{3+}) of the synthetic AMD and the ratio between DW and AMD were investigated in batch assays. Since SRB lower their activity at pH<5 (2), the election of Fe^{2+} over Fe^{3+} depended on the final pH of the AMD:DW mixture, which turned out to be 5.6 with Fe^{2+} and over 2.9 with Fe^{3+}. After mixing AMD and DW, flocks appeared after several minutes due to the coagulant properties of metal cations, which cause the co-precipitation of suspended and colloidal organic matter. This fact causes organic matter loss, so the theoretical calculated AMD:DW ratio (1:7) was increased up to 1:10. Additionally, analyses of metal concentration showed a reduction of metal concentration due to coagulation (%): Fe 51.5, Mn 31.4, Co 38.7, Ni 49.2, Cu 68.2 and Zn 34.1, which indicates that the mere mixture of DW and AMD started the remediation process chemically.

Water quality

R1 and R2 showed similar evolution patterns for COD, sulfate and total Fe removal throughout the experiment (Fig. 2). However, the efficiencies were slightly higher for R1 than R2 during phase I to III (COD, sulfate and Fe removal: 62%, 39%, 51% for R1 and 46%, 25% and 41% for R2). Significant fluctuations in the removal efficiencies were observed during these phases, possibly due to the variations of the DW influent. As observed, the mean COD removal efficiencies were 62% and 46% for R1 and R2, even with electron acceptor excess, which might reflect the non-degradable nature of some compounds of the domestic water. Supporting this is the fact that after acetate supplement reduction efficiencies increased notably for the three parameters (88, 75 and 85%), and became more stable. According with these results, a fraction of the DW organic matter (24±3%) is scarcely biodegradable, limiting sulfate reduction. For this reason, sulfate reduction efficiency seems to be coupled to the COD/SO_4^{2-} ratio (1:1) more than the theoretical ratio 0.67:1.

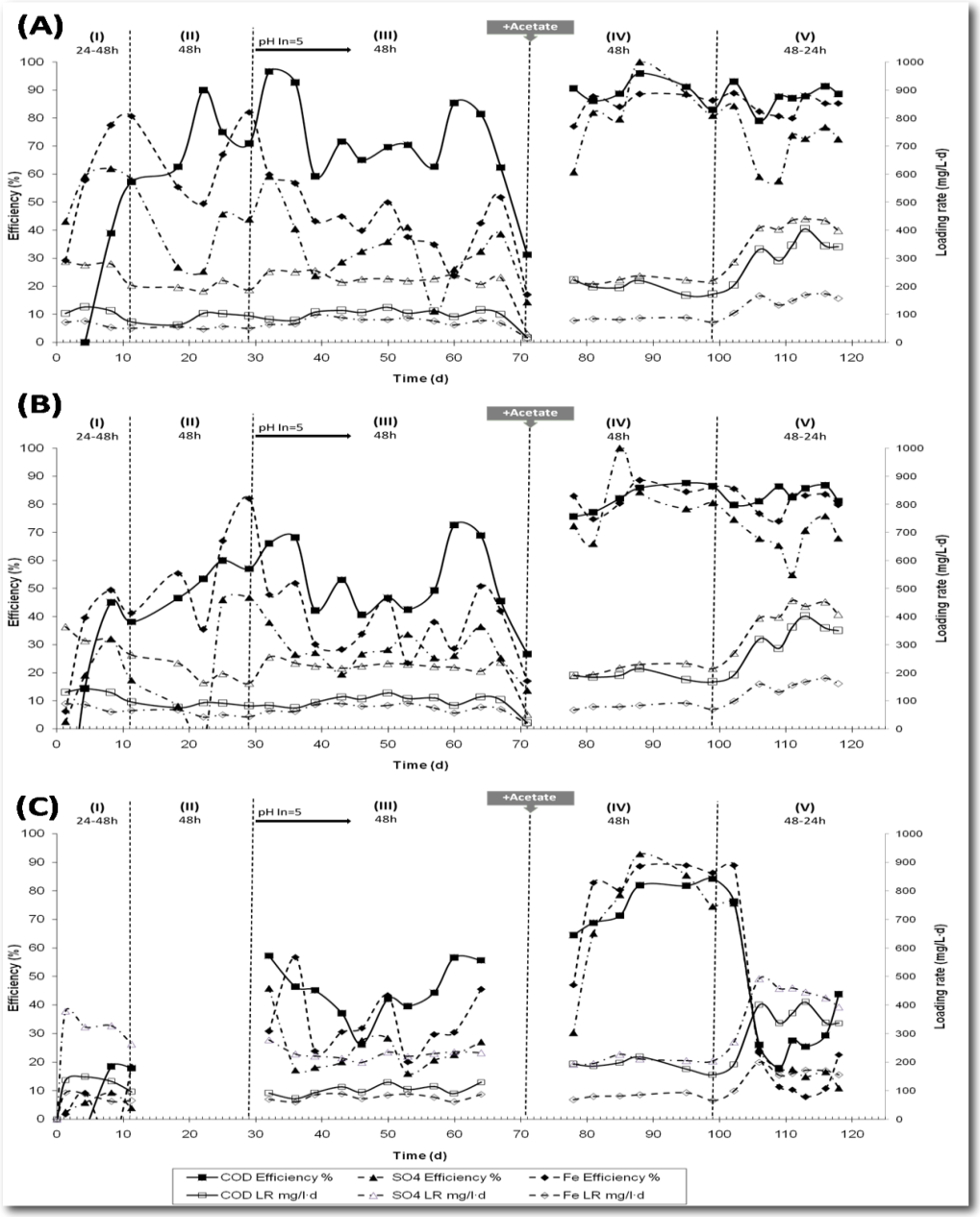


Fig. 2: Removal efficiency (%) and loading rates (mg L^{-1}·d^{-1}) of COD, sulfate and Fetot for R1-AC (A), R2-sediment-agar (B) and R3-liquid culture-agar (C).

The removal efficiencies for R1 and R2 were similar during phases IV and V, thereafter, the highest removal rates were achieved at phase V when HRT was decreased from 48 to 24 h. Removal rates were very close for the two reactors with values as high as 350 mg-COD L^{-1}·d^{-1}, 340 mg SO_4^{2-} L^{-1}·d^{-1} and 150 mg Fe L^{-1}·d^{-1} for total Fe. The reduction of HRT did not affect the COD and Fe removal efficiency. Only sulfate reduction efficiency decreased from 82 to 68 %. The sulfate reduction rates observed were similar to those reported by Greben and Maree (8),although they were lower than those seen in other studies of AMD bioremediation at low pH (8, 12-13). This can be explained by the use of a complex source of organic matter, which is not readily available for the SRB, since the studies before mentioned used simple organic substrates. Moreover, R1 and R2 were performing well, thus, HRT could have been lowered.

Since the performance of R3 during phase I was very low, the reactor was re-inoculated with fresh inoculum (days 11 to 32). The highest removal rates were achieved during phase IV, with

removal efficiencies that were slightly lower than the ones for R1 and R2. However, at phase V, R3 efficiencies dropped drastically. The overloading of the reactor, which is confirmed by a decreased of the pH, or diffusion limitation of the nutrients inside the cell, suggested by SEM observations, could be the reasons.

Throughout the experiment, an increase of the pH effluent was observed in the three reactors. This increase is caused by the inorganic carbon formed during anaerobic respiration (12). Effluent pH stayed at approximately 6.0 for the three reactors through phases I to III. After the addition of acetate, pH values increased up to 6.3 and were more stable, except for R3 at phase V when pH dropped to 5.7.

Between days 64 to 78 the accumulation of precipitates at the reactor inlets caused an obstruction in the pipes. This is a common problem in systems in which sulfate reduction and metal precipitation occur in the same reactor (17). The separation of these processes in two different reactors may reduce clogging problems (2, 5, 15).

Metal removal

Metal removal efficiencies were elevated (above 99%) for Co, Ni, Cu and Zn and Fe for R1 and R2, releasing an effluent with low metal concentrations. The performance of R3 was also very satisfactory for metal removal (higher than 98%) with the exception of Fe (Table 2). Mn removal was low for the three reactors, even reaching negative values, which can be explained by an accumulation in the reactor during previous phases. Poor performance of Mn removal is explained by its chemistry: Mn has to be oxidized from Mn^{2+} to Mn^{4+} before being removed as MnO_2 (10).

Table 2: Average metal concentration (mg L^{-1}) and removal efficiency (% in brackets) for phases IV and V for the three reactors (R1, R2 and R3).

	Average metal concentration (mg L^{-1}) and removal efficiency (%) for each metal											
	Fe		Mn		Co		Ni		Cu		Zn	
R1	13.2 ±10	85	2.5 ±0.8	7.7	$5x10^{-4}$ $\pm5x10^{-4}$	99.9	$1.3x10^{-3}$ $\pm8x10^{-4}$	99.1	$1.4x10^{-2}$ $\pm1x10^{-2}$	99.1	$4.9x10^{-2}$ $\pm2x10^{-3}$	99.5
R2	12.7 ±9.2	85.2	3.4 ±0.4	-13	$5x10^{-4}$ $\pm5x10^{-4}$	99.9	$8x10^{-4}$ $\pm4x10^{-4}$	99.4	$8.5x10^{-3}$ $\pm8x10^{-3}$	99.5	$5.2x10^{-2}$ $\pm2x10^{-3}$	99.4
R3	42.1 ±41	49	3.4 ±0.8	-13	$2x10^{-4}$ $\pm2x10^{-4}$	99.6	$3x10^{-3}$ $\pm3x10^{-3}$	98.0	$7x10^{-3}$ $\pm5x10^{-3}$	99.5	0.11 ±0.07	98.9

DGGE

The microbial community and its variation throughout the experiment were analyzed by DGGE. Comparing the band pattern from time 0 and the end of phases III and IV (days 67 and 118), an increase in the microbial diversity in the samples from the reactor is observed (Fig. 3) possibly due to a diversification of the community to adapt to a complex source of nutrients, like DW, and the colonization of carriers by bacterial species present in the DW.

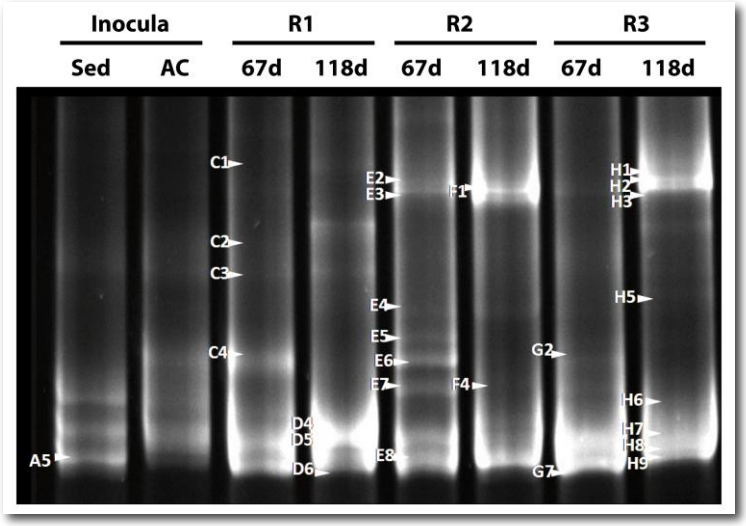


Fig. 3: DGGE fingerprints from the inocula (Tinto Sediment and enrichment culture with Activated Carbon), and the three reactors' supports at day 67 (end phase III) and day 118 (end phase IV).

The sequences obtained (Table 3) fell into two main metabolic bacterial groups: (i) fermentative bacteria, which would metabolize complex substrates: *Clostridium* spp., *Paludibacter* spp. and *Pelotomaculum* spp. (syntrophic bacterium) and (ii) SRB, which would use the intermediate molecules produced by fermenters or the added acetate: *Desulfomonile* spp., *Desulfovibrio* spp., *Desulfotomaculum* spp. and *Desulfosporosinus* spp. *(Clostridia)* and *Desulfurella* spp. (*Deltaproteobacteria).*

Several bacterial sequences identified in this study have a high similarity to those identified in a recent study of Tinto River sediments (20). For instance, bands E2, E3, F1, H1, H2, H3 share a high similarity with HQ730741 (uncultured *Paludibacter* sp. detected by DGGE) and band A5 shares a high similarity with HQ730647 (uncultured *Peptostreptococcaceae* identified by cloning). These organisms were found in those sediment strata with reducing redox potential and less acidic pH (5.3-5.4). Other bands (E4, E5, E6, E7) are related with HM745405 (*Desulfosporosinus* spp.), a sequence retrieved from a close area of the Iberian Pyritic Belt (6). Bands F4 and H8, identified in R2 and R3 at day 118, share a high similarity with FJ873799 (*Desulfovibrio desulfuricans* strain BSR-22). This organism has been reported as the predominant SRB species in a sulfate reducing bioreactor working at pH 4-4.5 (1). Furthermore, other identified bacteria, for instance bands D4 and D5 seem to be related with AY167450 (uncultured *Desulfomonile*) found in an acidic environment.

Table 3: DGGE sequenced bands and their closest related named gene sequences in the NCBI database

Lane	Band	Closest related species	NCBI Accession number	Identities (%)
Tinto Sedim	**A5** (JQ517512)	Uncultured *Peptostreptococcaceae* clone JL22_2009	HQ730647	94
		or *Clostridium* sp. K39	AB610575	94
R1 T1	**C1** (JQ517513)	*Clostridium acetobutylicum* (NCP 262)	X78073	94
	C2 (JQ517514)&		AB610548	97-96
	C3 (JQ517515)& **C4** (JQ517516)	*Clostridium diolis*		100
R1 T2	**D4** (JQ517517)&	Uncultured *Desulfomonile* (Fen soil)	AY167450	95-92
	D5 (JQ517518)	or *Desulfomonile tiedjei*	AM086646	92-89
	D6 (JQ517519)	Uncultured sulphate-reducing bacterium (Benzene-degrading enrichment culture)	EU523095	97
		or *Pelotomaculum schinkii*	X91170	97
R2 T1	**E2** (JQ517520)&	Uncultured *Paludibacter* sp. DGGE gel band JL5a	HQ730741	97
	E3 (JQ517521)		CP002345	90
		or *Paludibacter propionicigenes*		
	E4 (JQ517522)&	Uncultured bacterium (AMD, Iberian Pyritic Belt)	HM745405	97
	E5 (JQ517523)&		AJ306703	97
	E6 (JQ517524)&	or *Desulfosporosinus* sp. ML1/10-BSR14		
	E7 (JQ517525)			
	E8 (JQ517526)	*Desulfomonile tiedjei*	AM086646	88
R2 T2	**F1** (JQ517527)	Uncultured *Paludibacter* sp. DGGE gel band JL5a	HQ730741	96
	F4 (JQ517528)	*Desulfovibrio desulfuricans* strain BSR-22	FJ873799	98
R3 T1	**G2** (JQ517529)	*Desulfosporosinus* sp. ML1/10-SRB3	AJ306705	93
	G7 (JQ517530)	*Desulfotomaculum* sp. ECP-C5	AF529223	92
R3 T2	**H1** (JQ517531)&	Uncultured *Paludibacter* sp. DGGE gel band JL5a	HQ730741	99
	H2 (JQ517532)		CP002345	89
		or *Paludibacter propionicigenes*		
	H3 (JQ517533)	Uncultured *Paludibacter* sp. DGGE gel band JL5a	HQ730741	100
			CP002345	91
		or *Paludibacter propionicigenes*		
	H5 (JQ517534)	*Delftia* sp. R-41380	FR682925	97
	H6 (JQ517535)	16S rDNA sequence from human fecal sample	FP083728	94
		or *Desulfovibrio piger* ATCC 29098	NR_041778	93
	H7 (JQ517536)	*Desulfovibrio* sp. DSM12803	AJ251630	93
	H8 (JQ517537)	*Desulfovibrio desulfuricans* strain BSR-22	FJ873799	91
	H9 (JQ517538)	*Desulfovibrio desulfuricans* strain Ser-1	EU980605	95

SEM

Samples from the three reactors were examined through SEM at the start and end of the experiment. In R1, colonization, with cocci and coccobacili as predominant organisms, was observed over the surface of the activated carbon used as a support (Fig. 4a). At the end of the experiment, surface images showed the formation of a dense biofilm (Fig. 4b). In R2, Tinto River sediments used as inoculum showed, from beginning to end, little colonization and non-biofilm formation (Fig. 4c). Figure 4d shows the inner part of the bacterial support in R3 at the end of the experiment. An electron back-scattered filter was used to differentiate between organic and metallic structures showing that cells were coated with metallic compounds. Metal sulfides could have precipitated onto bacterial cell walls, isolating them from the surrounding environment and preventing the access of reactants to the cell (20). This may have been the reason for the poor performance of R3 throughout the experiment and the cause for the collapse in phase V. This phenomenon, called encapsulation (19), may have also occurred in R1 and R2, however either bacterial growth could have overcome the rate of cell encapsulation or a better inner structure may have allowed internal bacterial development protected from the outer metal precipitation.

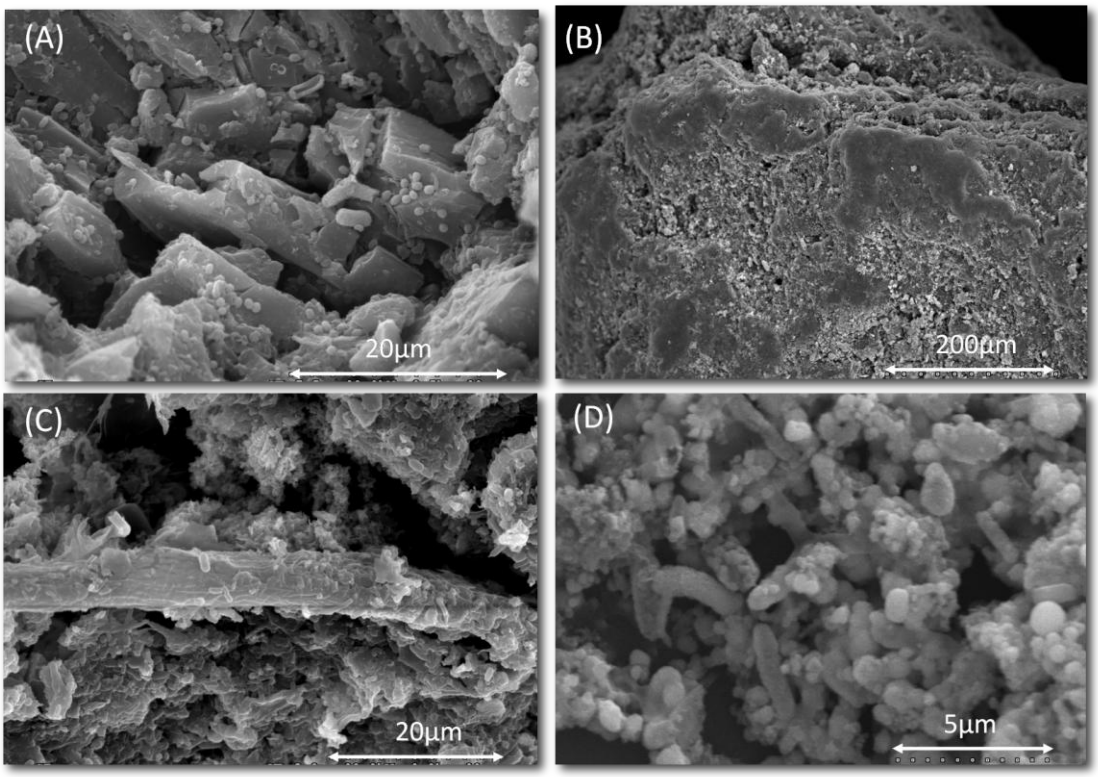


Fig. 4: SEM images of the bacterial supports. Bacterial colonization of the activated carbon in R1 at the beginning of the experiment (A) and at the end (B). Sediment used as inoculum in R2 (C). Cell structures coated with metal precipitates in the inner part of the support of R3 (D).

CONCLUSIONS

Results show that it is possible to bio-remediate AMD by its co-treatment with DW in anaerobic bioreactors. Around half of the dissolved metal ions were chemically removed along with organic matter just by mixing ARD and DW. Good heavy metal removal efficiencies were achieved during the operation. Additional substrate supply

made elevated pollutant removal efficiencies possible (COD>88 %; sulfate>75 %; Fe_{Tot} >85 %; and dissolved metals >99 % except Mn) leading to a good effluent quality. No significant differences were found between R1 and R2, but the agar matrix used for R3 was the least efficient, collapsing in the last phase of the experiment. Two groups of bacterial metabolism were found by DGGE: fermenters and SRB. Clogging problems and the encapsulation phenomenon can be important problems when sulfate reduction and metal precipitation occur in the same reactor.

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DISCUSIÓN GENERAL

En cada uno de los capítulos que componen la presente tesis se ha llevado a cabo una exhaustiva discusión de sus resultados. En este apartado se procura hacer una puesta en común de los mismos para obtener una visión global de la geomicrobiología de los sedimentos del río Tinto, combinando los datos de diversidad microbiana obtenidos mediante técnicas de ecología molecular y clásica con los datos físico-químicos de los sedimentos.

BIOGEOQUÍMICA GENERAL DE LOS PUNTOS DE MUESTREO

Atendiendo a criterios geo-edafológicos y climáticos, el cauce del río Tinto, desde su origen en Peña de Hierro hasta su desembocadura en la ciudad de Huelva, se divide en tres zonas principales: curso alto o zona norte, curso medio o zona de transición y curso bajo o estuario (82). La presente tesis se ha centrado en la zona norte (desde Peña de Hierro a La Palma del Condado) (Fig. 1), consistente en un complejo volcánico paleozoico donde las aguas del río presentan las características propias del Tinto: altas concentraciones de ión férrico en disolución y un pH ácido, cercano a 2,3.

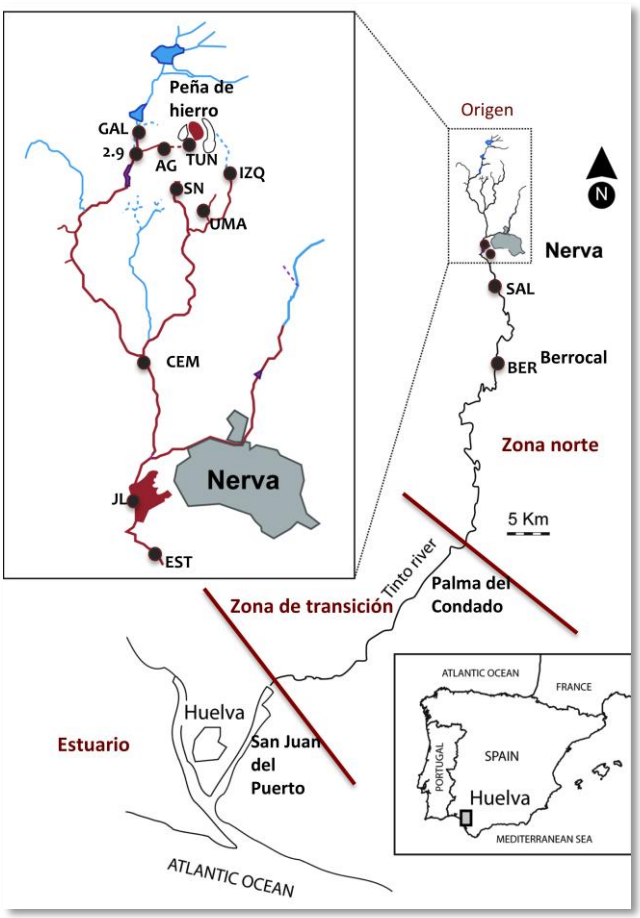

Figura 1: Mapa del río Tinto. Zonas de división y la localización de los puntos de muestreo.

Todos los puntos de muestreo se seleccionaron en el curso del río que transcurre por la zona norte. Cercano al origen, a la salida del túnel de la corta de Peña de Hierro se encuentra el punto denominado Túnel (*TUN*), con aguas ferrosas con bajo contenido en oxígeno. En su recorrido, el agua se va oxigenando y los componentes minerales oxidándose hasta llegar al jardín de Anabel (*AG*), en la que el agua ya presenta las típicas características del Tinto, con bajo pH y alto contenido en hierro en su forma oxidada. Este arroyo confluye con otro donde

se sitúa Campo de Galdierias (*GAL*), y aguas abajo se encuentra una represa bautizada como 2.9 (2.9). Por otro lado, al este de Peña de Hierro confluyen tres arroyos donde se sitúan la presa *SN*, *UMA*, e izquierda (*IZQ*). Ambos grupos de arroyos confluyen aguas abajo uniéndose en un punto cercano a cementerio (*CEM*). Unos metros más adelante, cercano al municipio de Nerva, se sitúa una presa cuyo punto de muestreo es conocido como *JL*, donde vierte el efluente de la depuradora municipal de Nerva y pocos metros después se encuentra el punto de muestro estación (*EST*). A partir de esta zona, se muestrearon dos estaciones: Salinas (*SAL*), denominado así por la construcción de balsas de evaporación destinadas a la recuperación del cobre en las terrazas del río y Berrocal (*BER*), próximo a la localidad del mismo nombre.

El río Tinto se muestra considerablemente homogéneo atendiendo a parámetros como pH y potencial redox, mostrando condiciones ácidas y oxidantes a lo largo de su curso gracias al efecto tampón que ejerce el férrico (61). Sin embargo, la adición de afluentes neutros genera un gradiente decreciente en la concentración de metales (especialmente hierro) y, consecuentemente, también un gradiente en la conductividad, que se pone de manifiesto - en la zona muestreada - en una disminución gradual desde los orígenes hasta el punto de muestreo *BER*. Destacan ciertos puntos de muestreo como *TUN*, cercano al origen, donde las condiciones anóxicas todavía no han generado las características extremas que se presentarán aguas abajo mostrando bajo potencial redox, bajo contenido en sulfatos y baja conductividad; *UMA,* donde aparecen los menores valores de pH y los mayores de potencial redox, conductividad y arsénico; o CEM, con los mayores valores de Ni, Cu y Zn, posiblemente debido a la influencia de un gran deposito cercano de gossan.

El lecho del río Tinto es rocoso por lo que la mayoría de los puntos de muestreo presentan acumulaciones carentes de estructura real de sedimento: consisten en partículas minerales de cierto tamaño como arenas y grava con alta porosidad. Sus características biogeoquímicas están fuertemente influenciadas por las de la columna de agua y por tanto lo están sus comunidades microbianas. Así, en estas acumulaciones encontramos comunidades pertenecientes principalmente a hábitats óxicos aunque algunos microorganimos pertenezcan a ambientes anóxicos. Por ejemplo, en distintas localizaciones encontramos oxidadores de hierro comunes de la columna de agua como la actinobacteria acidófila heterótrofa *Ferrimicrobium,* el quimiolitótrofo aerobio estricto *Leptospirillum,* o el anaerobio facultativo *Acidithiobacillus ferrooxidans*, así como diversos géneros de heterótrofos aerobios estrictos como *Pseudomonas*, *Pedobacter*, *Flectobacillus*, etc. También encontramos fermentadores como *Clostridium* spp. y, en menor medida, anaerobios estrictos como bacterias sulfato-reductoras (BSR) de los géneros *Desulfosporosinus* o *Desulfobulbus*.

Sin embargo, existen ciertas zonas con pequeñas presas – la lámina de agua raramente alcanza el metro de profundidad - que permiten la acumulación estratificada de sedimentos con menor tamaño de partícula, principalmente limos y arcillas. A estas zonas pertenecen los puntos *JL* y *SN* (Fig. 2). En ellos, la menor porosidad y la estratificación de los sedimentos permite cierta diferenciación de sus características físico-químicas respecto a las de la columna de agua, encontrándose verdaderas condiciones de anaerobiosis.

Figura 2: Vista de los principales puntos de muestreo y de sus testigos tipo: (a) presa SN y (b) presa JL.

Mientras en la presa *SN* los sedimentos aparecen más homogéneos, tanto espacial como temporalmente (Fig. 2a), en la presa JL la estratificación ha sido observada repetidamente, aunque variando temporalmente la localización, grosor y parámetros de los estratos (Fig. 2b). En JL generalmente aparecen dos zonas principales, una de aspecto marrón con valores de pH que varían en torno a 4 y potencial oxidante (hasta 168 mV) y otra zona negruzca con valores de pH alrededor de 5,5 y potenciales redox reductores (hasta -246 mV).

En estas presas la estratificación permite una diversificación de nichos ecológicos, desarrollándose una comunidad microbiana mayoritariamente anaerobia, distante de la encontrada en la columna de agua y vinculada con los parámetros físico-químicos de los sedimentos. En *SN*, con pH y potencial redox más cercanos a la columna de agua (pH~2,5 y E~+400 mV), siendo el hierro un aceptor de electrones preferente por su solubilidad a bajo pH, aparecen *At. ferrooxidans* y *Acidiphilium* spp. cuyo metabolismo regenera el ión ferroso. En cambio, en la presa *JL* con alta variabilidad físico-química, los organismos varían desde *Acidobacteria* en los estratos más oxidantes y ácidos, a organismos fermentativos como *Paludibacter* spp. y *Clostridium* spp. en condiciones menos oxidantes, hasta sulfato-reductores como *Desulfosporosinus* y *Desulfurella* en aquellos estratos más reductores y de mayor pH.

Los datos de clonaje, DGGE y CARD-FISH (83, 84) evidencian la enorme diferencia entre las comunidades microbianas de las presas *JL* y *SN*, tanto en número como en composición, reflejando sus diferencias geoquímicas. La presa *SN* está localizada en la cabecera del río con un caudal constante con alta concentración de férrico que tampona no solo los parámetros del agua sino incluso los de los sedimentos. Sin embargo, JL está situado 5 km aguas abajo, con un caudal mayor -debido a la adición de afluentes neutros que rebajan la concentración de hierro- y variable –debido a la estacionalidad del clima mediterráneo al que están sometidos los afluentes. Uno de los afluentes que vierten aguas arriba de *JL* proviene de la estación depuradora de aguas residuales de Nerva, lo que incrementa notablemente el contenido de materia orgánica. La combinación de ambos factores podría explicar las diferencias detectadas en ambos tipos de sedimentos. En JL, con variabilidad estacional, mayor contenido en materia orgánica y menor concentración en hierro, ciertos procesos reductivos microbianos, como la sulfato-reducción, podrían aumentar el pH y reducir el potencial redox. Este efecto comenzaría en micronichos en el sedimento y poco a poco se expandiría a condiciones macroscópicas. Esto

explicaría el sedimento bandeado en JL, que podría estar relacionado con fluctuaciones del agua superficial.

La diversidad encontrada en los sedimentos del río Tinto ha demostrado ser mucho mayor que la encontrada en la columna de agua en estudios previos (29, 32). Mediante técnicas moleculares, como clonaje y DGGE, a lo largo de este trabajo se han identificado directamente de sedimentos secuencias pertenecientes a 12 *phyla* bacterianos distintos: *Proteobacteria* (clases *Alpha-, Beta-, Gamma- y Delta-proteobacteria*), *Firmicutes*, *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, *Synergistetes, Cyanobacteria/Chloroplast, TM7, Chloroflexi, Nitrospira, Verrucomicrobia, Fibrobacter.* Las secuencias han podido ser afiliadas filogenéticamente a 72 géneros diferentes. Respecto a arqueas, prácticamente todas las secuencias correspondían a los géneros *Ferroplasma* y *Thermoplasma* (orden *Thermoplasmatales, phylum Euryarchaeota*); a excepción de una secuencia de *Methanosaeta concilii (Euryarchaeota)* y la familia *Thermofiliaceae* (*Crenarchaeota).*

Dada la diversidad encontrada y la complejidad de los posibles metabolismos que interactúan en este ecosistema, es necesaria una discusión global de los resultados repasando los distintos ciclos geoquímicos encontrados. Debido al substrato geológico por el que transcurre la zona norte del río Tinto, el hierro y el azufre - en sus distintas formas moleculares - están presentes en altas concentraciones en el ecosistema. Sin embargo, y en contraste con lo que pudiera suceder en las aguas, donde dominan los metabolismos implicados en el ciclo biogeoquímicos del hierro, en los sedimentos también se detecta una amplia actividad relacionada con los ciclos del N y del C.

CICLO DEL HIERRO

El hierro suele ser un elemento limitante en la mayoría de los ecosistemas ya que su baja solubilidad a pH neutro hace que sea poco biodisponible. Dado el substrato geológico que ofrece la FPI, y a la mayor solubilidad del hierro a bajo pH, el hierro se encuentra biodisponible a altas concentraciones (~2,3 g L^{-1}) en el ecosistema del Tinto.

La estabilidad del hierro ferroso a bajos pHs facilita su oxidación biológica al no competir con la oxidación química (94), permitiendo la abundante presencia y actividad de microorganismos acidófilos oxidadores de hierro. Nótese que a continuación se discute la capacidad de oxidación de hierro de los organismos, sin detrimento de que por su versatilidad puedan reducirlo u oxidar otros compuestos. La **oxidación desasimilativa** del hierro puede llevarse a cabo tanto por organismos autótrofos como heterótrofos. En las aguas del río Tinto otros estudios han demostrado el predominio absoluto de los oxidadores acidófilos extremos quimiolitótrofos como *At. ferrooxidans* o *Leptospirillum* spp. y la presencia del heterótrofo *Ferroplasma* spp. (30, 32, 61). Sin embargo, en este trabajo, técnicas moleculares como clonación han mostrado una mayor diversidad de oxidadores de hierro en los sedimentos del Tinto. Además de especies autótrofas (*At. ferrooxidans, L. ferriphilum* o *L. ferrooxidans,*), aparecen gran variedad de autótrofos facultativos (*Sulfobacillus* spp., *Alicyclobacillus* spp.), o heterótrofos obligados (*Ferrimicrobium* spp., *Ferrithrix* spp.). Esta gradación de comunidades oxidadoras corresponde a sus necesidades fisiológicas y requerimientos ecológicos. En los drenajes ácidos de mina, el oxidador aerobio obligado *Leptospirillum* domina las comunidades microbianas de las zonas óxicas con condiciones más extremas (78) debido a su preferencia por bajos pH, su mayor tolerancia a altas concentraciones de férrico y su mayor afinidad por el ión ferroso (K_m=0,025 mM) que *At. ferrooxidans* (K_m=1,34 mM). Sin embargo, a menores

potenciales redox, el anaerobio facultativo *At. ferrooxidans* posee mayor actividad específica oxidadora (44) compitiendo eficientemente. En los sedimentos óxicos del río, donde cierta acumulación de materia orgánica es posible, se incrementan los organismos heterótrofos. *Ferrimicrobium* y *Ferrithrix,* actinobacterias acidófilas extremas y heterótrofas obligadas (42), aparecerán en las zonas de menores pH mientras que *Alicyclobacillus* tiene preferencia por zonas de pH algo más elevado (63). También aparece *Sulfobacillus* spp., capaces de crecer de forma autótrofa, mixótrofa y heterótrofa (108). Finalmente, en los sedimentos anóxicos - donde hay limitación de oxígeno pero no de materia orgánica - los quimiolitótrofos y hetótrofos comparten nicho ecológico pero se limitan a aquellos con un metabolismo más versátil como *Sulfobacillus* spp., *Alicyclobacillus* spp. o *At. ferrooxidans,* que además es capaz de oxidar H_2 (21). Aunque estos organismos podrían utilizar el posible oxígeno que difundiera a los sedimentos, su persistencia parece atribuible a su versatilidad metabólica; al ser capaces de reducir hierro en ausencia de oxígeno, actuarían más probablemente como reductores que como oxidadores de hierro en estos sedimentos anóxicos.

Por último, la oxidación de hierro también podría aparecer acoplada a desnitrificación en condiciones anóxicas (93). Diversos estudios han detectado esta capacidad en cepas de *Pseudomonas*, bacterias púrpuras (25), y *Dechloromonas* (2, 14), todas ellas presentes en los sedimentos del Tinto. Además esta actividad ha sido detectada en cultivos de enriquecimiento con sedimentos del Tinto donde se apreciaba la oxidación de ferroso a férrico en condiciones de anaerobiosis.

La **reducción desasimilativa** del hierro está muy extendida entre los acidófilos, pudiendo ser llevada a cabo por una gran variedad de microorganismos. Numerosas secuencias pertenecientes a organismos hierro-reductores han sido detectadas por técnicas moleculares (DGGE y clonación) en los sedimentos del río Tinto; además, la reducción de hierro ha sido confirmada mediante medida de actividad en cultivos de enriquecimiento específicos, siendo mayor a menores pHs. Algunos de los reductores corresponden a los versátiles oxidadores de hierro antes mencionados. Entre ellos, *At. ferrooxidans* – capaz de reducir ión férrico usando azufre elemental (74) o hidrógeno (71) como dadores inorgánicos de electrones - parece tener un papel principal al aparecer de forma profusa en las librerías genéticas (84) y mostrar altos porcentajes de hibridación (17% respecto al número total de bacterias) en puntos como *SN* (83). Otros oxidadores de hierro, tales como *Ferrimicrobium* spp., *Ferrithrix* spp. (42), *Sulfobacillus* spp. (108), y *Ferroplasma* (20) son también capaces de reducir hierro usando compuestos orgánicos como dadores de electrones. *Alicyclobacillus* spp. son capaces de reducir hierro aunque no siempre vinculado al crecimiento (63).

Aparte de los anaerobios facultativos mencionados en el párrafo anterior, capaces de oxidar o reducir hierro, existen numerosos reductores de hierro especializados. En ausencia de oxígeno, el ión férrico es el aceptor preferencial en los sedimentos para la mineralización de la materia orgánica (13) dado que procesos como la sulfato-reducción y la metanogénesis se inhiben en su presencia (9). El heterótrofo *Acidiphilium cryptum* es capaz de reducir hierro oxidando una diversidad de compuestos orgánicos de bajo peso molecular, azúcares o hidrógeno (43), incluso en presencia de oxígeno (57). Este organismo, identificado en los sedimentos del Tinto, parece jugar un papel relevante en determinados emplazamientos, como SN donde representa el 5% del total de bacterias presentes en el mismo (83). Diversas acidobacterias también son capaces de reducir hierro (63), específicamente, *Acidobacterium capsulatum* que forma Fe(II) en condiciones anaerobias a pHs de 3 a 5 respirando glucosa (17).

También aparecen *Geobacter*, anaerobio estricto capaz de usar ácidos grasos de cadena corta, alcoholes y compuestos monoaromáticos como dadores de electrones (62) para la reducción de hierro; *Ferribacterium,* capaz de usar acetato u otros acidos orgánicos (18) y *Aciditerrimonas ferrireducens*, termoacidófila anaerobia (39). Por último, multitud de BSR, tales como *Desulfosporosinus* spp., pueden actuar como Fe (III)-reductoras (77). Estos procesos implican la regeneración del ión ferroso en los sedimentos del río Tinto completando el ciclo del hierro con los organismos encontrados.

Los metabolismos basados en el hierro están íntimamente relacionados con las transformaciones minerales. Aparte de la conocida acción de los oxidadores de hierro en la lixiviación de los sulfuros metálicos, los fenómenos de reducción también parecen estar implicados en otros procesos. Por ejemplo, se asocia la acción de BSR como *Desulfosporosinus* sp. GBSRB4.2 con la transformación de schwertmanita [$Fe_8O_8(OH)_6SO_4 \cdot nH_2O$] a goetita (α-FeOOH) al aumentar el pH y la concentración de Fe(II) (7) o la acción reductiva de *A. cryptum* JF-5 sobre el hierro contenido en minerales como goetita, jarosita, etc. favoreciendo su disolución (12).

CICLO DEL AZUFRE

En las zonas anaerobias de ecosistemas con gran cantidad de pirita y otros sulfuros metálicos se acumulan distintas formas reducidas del azufre (tiosulfato, tetrationato, azufre elemental, sulfuro, etc.) que pueden ser usadas como substratos por distintos organismos en **procesos oxidativos**. Algunos de los organismos identificados como oxidadores de hierro también son capaces de oxidar sulfuros (*At. ferrooxidans* o *Sulfobacillus* spp.). Adicionalmente, diversas especies de *Acidiphilium* (*A. acidophilum*, *A. cryptum*, etc.) pueden oxidar azufre elemental (34, 81). Este grupo de microorganismos podrían oxidar las especies reducidas de azufre con el oxígeno que difundiera a los sedimentos. Por último, se han identificado secuencias de cromatiales o bacterias rojas del azufre, que son organismos anaerobios o microaerófilos capaces de realizar la fotosíntesis. Éstos podrían usar el sulfuro de hidrógeno como agente reductor produciendo gránulos de azufre que pueden ser posteriormente oxidados totalmente hasta sulfatos.

Pese a que la forma mayoritaria y estable del azufre es el sulfato, hasta la fecha no son muchos los acidófilos conocidos capaces de catalizar la **reducción desasimilativa** del sulfato a sulfuro. Generalmente las BSR prefieren pHs entre 6 y 8 (103) pero, desde hace unos años, se ha visto que este metabolismo está presente en diversos ambientes ácidos (53), entre ellos río Tinto. Mediante técnicas moleculares, en la presente tesis se han identificado diversos géneros de BSR (*Desulfosporosinus*, *Desulfotomaculum*, *Desulfobulbus, Desulfosalsimonas, Desulfitobacterium* y *Thermodesulfobium*) y reductores de azufre elemental de los géneros *Thermoplasma, Acidithiobacillus* y *Desulfurella* en los sedimentos del Tinto (83-85).

La arquea termoacidófila anaerobia facultativa *Thermoplasma* (87) y la bacteria termoacidotolerante anaerobia *Desulfurella* (46), capaces de respirar azufre elemental, habían sido previamente detectadas en diversos ambientes ácidos. En recientes investigaciones se ha visto que varias especies de *Acidithiobacillus*, entre ellas *A. ferrooxidans,* son capaces de respirar azufre oxidando hidrógeno a pHs tan bajos como 2,5 (40). Entre las BSR, *Syntrophobacter* puede usar sulfato como aceptor de electrones oxidando propionato por la via methylmalonil CoA (36); el termófilo moderado autótrofo *Thermodesulfobium* puede usar hidrógeno (67); *Desulfosalsimonas* es una SRB halófila capaz de usar lactato, propionato,

acetato y butirato usando sulfato o tiosulfato como aceptores de electrones (52, 90); *Desulfobulbus* (105) usa propionato, lactato y alcoholes oxidándolos incompletamente a acetato, o bien, H_2 suplementado con acetato como fuente de carbono. *Desulfosporosinus* spp. pueden también fermentar distintos substratos (91), realizar acetogénesis a partir de formiato o metanol (75) o reducir hierro disasimilativamente (77). *Desulfosporosinus* spp. y *Desulfotomaculum* spp., oxidadores incompletos de diversos compuestos orgánicos a acetato, son anaerobios obligados con la habilidad de esporular, lo que les hace más resistentes a condiciones adversas pudiendo sobrevivir a condiciones oxidantes y de sequedad durante meses (104).

Mediante técnicas de hibridación *in situ* se ha visto que los géneros *Desulfosporosinus*, *Desulfurella* y *Syntrophobacter* son muy abundantes (11, 6 y 5% del número total de bacterias) en ciertos estratos de los sedimentos (83), especialmente en aquellos de mayor pH (4,2-6,2) y condiciones redox mas reductoras (50, -210 mV), llegando sólo *Syntrophobacter* a representar el 37% del total en el estrato superficial. El número de BSR se correlaciona con la variación de los parámetros físico-químicos de los sedimentos. Donde las BSR son más abundantes, como consecuencia de su metabolismo, se reduce la concentración de sulfato y aumenta el pH. El sulfuro de hidrógeno producido reacciona con el hierro y otros metales precipitándolos en forma de sulfuros. De este modo, se observa una atenuación natural, llevada a cabo por las BSR, de las características extremas de ambientes ácidos (bajo pH y alto contenido en hierro, sulfato y metales pesados en las aguas).

Adicionalmente, se han usado cultivos de enriquecimiento específicos para probar no solo su presencia y abundancia, sino también la actividad sulfato-reductora en los sedimentos. En una primera aproximación se realizaron enriquecimientos a pH 5 y 7 obteniendo resultados positivos a ambos pHs (85). Sin embargo, el aislamiento sólo fue posible a pH 7, perteneciendo todos los aislados al género *Desulfotomaculum* spp., una conocida BSR neutrófila (104). Estos resultados están en la línea de otros trabajos, en los que partiendo de inóculos de ambientes ácidos se aislaron BSR neutrófilas (58, 60, 95). Desde hace tiempo, hay un interés creciente en el aislamiento de BSR realmente acidófilas, pero con pocos resultados positivos. Otros autores consiguieron enriquecimientos que reducían sulfatos a bajo pH pero resultaron ser co-cultivos con *Acidocella* o *Desulfitobacterium* (15, 51). En algunos casos, se consiguieron aislar cepas acidófilas pero nunca fueron caracterizadas (35, 45). De modo que, tras todo el interés despertado por este metabolismo y el esfuerzo invertido, a día de hoy contamos con pocas cepas de verdaderas BSR acidófilas caracterizadas (4, 88). Por todo ello, se realizó un *screening* exhaustivo con sedimentos de río Tinto, obteniéndose cultivos que crecían a pH tan bajos como 4 con glicerol, metanol e hidrógeno, 4,5 con lactato y 5,5 con succinato. Además pudieron aislarse varias cepas, identificando dos nuevas especies del género *Desulfosporosinus* y un nuevo género cercano al grupo *Desufosporosinus*/*Desulfitobacterium* que crece a un pH óptimo de 5. Con este trabajo, se prueba la existencia y actividad de BSR en los sedimentos del río Tinto, además del carácter acidófilo y no sólo acidotolerante de los aislados.

Dado el carácter acidófilo de las comunidades presentes en el río y en vista de la atenuación natural de las características extremas de los ambientes ácidos de mina, observada en aquellos estratos donde eran abundantes las BSR, se planteó un posible uso tecnológico. Se acopló el tratamiento de aguas de drenaje ácido (con bajo pH y alta concentración de metales pesados, hierro y sulfatos) con el tratamiento de aguas residuales (alto contenido orgánico). El carácter acidófilo de las comunidades microbianas de los sedimentos del río Tinto fue clave, puesto que

con un inoculo de carácter neutrófilo hubiera sido necesario una neutralización del influente previa al tratamiento. Sin embargo, la comunidad sulfato-reductora intrínseca de los sedimentos del río Tinto fue capaz de llevar a cabo el tratamiento acoplado de ambos vertidos obteniendo en una sola etapa un efluente de calidad, a pH neutro y con bajo contenido en materia orgánica, sulfatos, hierro y metales pesados, mostrando la gran capacidad de biorremediación de las BSR de río Tinto.

CICLO DEL NITROGENO

Los ciclos del hierro y del azufre han sido profusamente estudiados en el río Tinto y ecosistemas similares, pero no se ha prestado ninguna atención al ciclo del nitrógeno en tales ambientes ácidos extremos, quedando su análisis excluido de revisiones globales (44). Sin embargo, durante la presente tesis se han detectado secuencias relacionadas con organismos que podrían estar involucrados en el ciclo del nitrógeno, se han medido actividades desnitrificantes en cultivos de enriquecimiento y se han detectado nitratos y nitritos en las aguas del Tinto. Por ello, se plantea el ciclo del nitrógeno como un ciclo activo e importante en el ecosistema.

Por técnicas moleculares se han identificado organismos con **capacidad fijadora de nitrógeno** tales como el quimiorganótrofo aerobio *Ideonella azotifigens* (70) o *Anaerobacter polyendosporus*, fermentador anaerobio obligado esporulante con capacidad de reducir nitrito a amonio y de fijar N_2 (23, 64). Además ha sido descrita la capacidad fijadora de diversas cepas de *Leptospirillum* spp. aisladas de río Tinto (30).

La **capacidad desnitrificante** está ampliamente extendida entre los organismos procariotas. El nitrato sirve de aceptor de electrones a multitud de bacterias heterótrofas y, en ocasiones, también autótrofas capaces de oxidar hierro ferroso, hidrógeno o compuestos reducidos de azufre. Por medio de clonaciones se han identificado géneros que contienen especies denitrificantes como *Alcaligenes*, *Pseudochrobactrum*, *Pseudomonas*, *Bacillus, Dechloromonas*, *Propionibacterium*, *Acidovorax, Delftia, Commamonas, Aquabacterium, Clostridium, Geobacter, Anaerobacter,* etc. presentando distintas tolerancias a condiciones óxicas.

Algunos de ellos son respiradores aerobios con capacidad de usar nitrato como aceptor de electrones alternativo, tales como *Acidovorax*, adaptados a ambientes oligotróficos y frecuentes en suelos contaminados con uranio (3, 50), *Delftia* (102), *Steroidobacter denitrificans* (26), *Comamonas aquaticus* (101), *Aquabacterium* que usa un amplio rango de carbohidratos (47), *Dechloromonas* (2, 14) y *Alicyclobacillus,* oxidadores aerobios de hierro y/o azufre capaces de usar nitrato como aceptor (41). También aparecen diversos respiradores de carácter anaerobio como *Alcaligenes faecalis,* capaz de respiración anaerobia con nitrito pero no con nitrato (73), *Geobacter,* que pueden oxidar acetato (62), o *Aquabacterium,* microaerofilos que oxidan un amplio rango de ácidos orgánicos pudiendo usar el nitrato como aceptor (47). Por ultimo, se han identificado fermentadores como *Brevibacterium* (16), *Pseudochrobactrum asaccharolyticum* (49), *Anaerobacter* (23, 64) y *Propionibacterium,* de lento metabolismo que producen lactato, propionato y acetato a partir de glucosa y capaces de desnitrificar (79).

La actividad desnitrificante en los sedimentos de río Tinto se confirmó mediante enriquecimientos específicos a distintos pHs (4,5 - 7), obteniendo resultados positivos en condiciones tanto heterótrofas como autótrofas. Esta actividad es dependiente de pH: mientras la reducción de nitratos se incrementa a pH más altos, la proporción de oxido nitroso

acumulado es mayor a menores pH. Esto confirma diversas investigaciones previas que sugieren que el rango óptimo de pH para la desnitrificación se sitúa entre 6 y 8; por debajo de éste, los pasos finales de la desnitrificación hasta nitrógeno molecular se inhiben, acumulándose los productos intermedios como nitritos (8, 69) u óxidos nitrosos (89). Mediante análisis por DGGE de dichos cultivos de enriquecimiento, se obtuvieron secuencias de organismos pertenecientes a géneros como *Bacillus*, *Paenibacillus*, *Lysinobacillus*, *Ammoniphilus*, *Rummelibacillus* (clase *Bacilli*); *Clostridium*, *Desulfitobacterium, Sedimentibacter* (clase *Clostridia*), y *Alcaligenes* (clase *Betaproteobacteria*), todos previamente vinculados a actividades desnitrificantes (98, 99). El análisis de diversidad de los microorganismos presentes en los cultivos confirmó la presencia y actividad de organismos previamente detectados mediante clonación y aportó evidencias de la participación de nuevos géneros con metabolismo desnitrificante en la cuenca del Tinto.

CICLO DEL CARBONO

En los estudios previos sobre las zonas aerobias de ecosistemas tipo drenajes ácidos de minas, el ciclo del carbono aparecía principalmente vinculado a, y dependiente de, los otros ciclos biogeoquímicos. La heterotrofía parecía tener un papel ecológico de mera detoxificación para los quimiolitótrofos, sensibles a altas concentraciones de materia orgánica (6). En cambio, durante la presente tesis, en los sedimentos del río Tinto se han obtenido secuencias y medido actividades vinculadas a diversos metabolismos del ciclo del carbono tales como oxidación heterótrofa, fermentación, metanogénesis e incluso acetogénesis, lo que evidencia un activo e importante ciclo del carbono operando en los sedimentos del río Tinto.

Atendiendo al modo de asimilación del carbono, los organismos se podrían dividir en autótrofos –aquellos que fijan CO_2 por mecanismos como el ciclo de Calvin (*At. ferrooxidans* o *Leptospirillum* spp.) - o heterótrofos, que asimilan el carbono contenido en compuestos orgánicos. En esta sección nos centraremos en discutir aquellos metabolismos centrados exclusivamente en el carbono, excluyendo aquellos heterótrofos que obtienen energía mediante respiración anaerobia utilizando compuestos de azufre, hierro o nitrógeno, los cuales se discuten en sus correspondientes secciones.

Algunas secuencias obtenidas corresponden a organismos heterótrofos cuya forma principal de obtención de energía es la **oxidación aerobia** de la materia orgánica. Entre ellos hay bacterias metilótrofas (oxidadoras de metanol) pertenecientes al género *Methylotenera* (48), además de multitud de organismos capaces de oxidar compuestos orgánicos más complejos (*Acidiphilium*, *Aquabacterium*, *Pedobacter*, *Flectobacillus*, *Sphingomonas*, *Variovorax*, *Pseudomonas*, etc.). Uno de los principales heterótrofos encontrados en la cuenca del Tinto, tanto en la columna de agua (32) como en sedimentos, es *Acidiphilium,* bacterias acidófilas extremas que oxidan compuestos orgánicos en condiciones aerobias o microaerófilas (43), llegando a representar un 5,6% de media respecto al número total de bacterias (83). En los sedimentos microaerófilos aparecen multitud de respiradores aerobios como *Aquabacterium* spp. que crecen en condiciones de microaerofília oxidando un amplio rango de ácidos orgánicos (47); *Pedobacter,* quimiorganotrofos con metabolismo oxidativo (92); *Haliscomenobacter*, bacterias filamentosas presentes en fangos activos que promueven la floculación (68); *Flectobacillus* aerobios obligados (59) al igual que *Acinetobacter*; *Rothia* o *Fluviicola* (72). U otros como *Leucobacter* con varias cepas resistentes al cromo (66); *Sphingomonas*, quimioheterótrofos aerobios obligados (28), *Dellvibrio*, bacterias celulolíticas

(65), *Pseudomonas*, bacilos aerobios no esporulantes (31) y *Cohnella*, aerobias formadoras de esporas. También fueron encontradas secuencias relacionadas con organismos facultativos como *Staphylococcus* y *Variovorax paradoxus*, un heterótrofo o autótrofo facultativo (oxidador de hidrógeno) capaz de usar una amplia variedad de compuestos orgánicos como fuente de carbono (106).

También se han identificado diversos organismos **fermentadores** como las bacterias del ácido láctico microaerotolerantes *Olsenella* (55), *Brevibacterium*, quimioorganotrofos aerobios estrictos, que oxidan y fermentan azúcares (16); *Propionibacterium*, fermentadores que, a partir de glucosa, producen acetato, lactato y propionato (19); *Prosthecobacter,* heterótrofos oligotróficos o *Fibrobacter*, bacterias celulolíticas que producen formato, acetato y succinato. También fermentadores anaerobios estrictos como *Turicibacter* (10), *Anaerobacter* spp., (23) fermentadores esporulantes de carbohidratos que producen etanol, acetato, lactato, butirato, butanol, hidrógeno y CO_2 (64); *Dorea*, quimiorganótrofos que producen etanol, formato, acetato, hidrógeno y CO_2 como producto del metabolismo de la glucosa; *Veillonella*, fermentadores de lactato que producen propionato, acetato, hidrógeno y CO_2 pudiendo usar también usar ácidos pirúvico, oxalacético, málico, fumárico, y succínico (80); *Propionispora hippie,* formador de esporas productor de propionato (1) o *Oscillibacter valericigenes,* productor de valerato (38). Muchos de estos fermentadores han aparecido en librerías genéticas amplificando DNA ambiental o DNA de cultivos de enriquecimientos de BSR a bajo pH donde, además, se han detectado algunos de sus productos de fermentación como butirato, propionato e hidrógeno. Esto confirma su presencia en los sedimentos de río Tinto y su actividad en las condiciones extremas que éstos presentan.

Uno de los fermentadores encontrado con mayor frecuencia mediante clonación, además de representar al menos el 1% de las bacterias totales en los sedimentos del Tinto, ha sido *Clostridium* spp. (83, 84). A pesar de ser bacterias anaerobias obligadas, no todos tienen la misma sensibilidad al oxígeno. Llevan a cabo la fermentación oscura, o fermentación ácida, en la que los sustratos orgánicos como azúcares o proteínas son fermentados a diversos ácidos orgánicos (butirato, acetato, propionato o lactato), alcoholes (etanol), CO_2 e hidrógeno (37, 97, 100). Por su capacidad de producción de hidrógeno fueron los organismos dominantes en los cultivos específicos de enriquecimiento para detectar tal actividad, habiéndose aislado varias cepas pertenecientes a este género. Estos aislados han sido caracterizados y está estudiándose su utilización en la producción biológica de hidrógeno (76).

Otro fermentador que parece jugar un papel importante en ambientes ácidos es un organismo lejanamente relacionado con *Paludibacter propionicigenes*, una bacteria anaerobia estricta productora de propionato (96). Numerosas secuencias relacionadas con este organismo fueron obtenidas por DGGE en estratos reductores de los sedimentos, así como en diversas clonaciones (84). Este fermentador fue detectado como el contaminante mayoritario en cultivos de enriquecimiento para BSR acidófilas, a partir de los cuales fue aislado por filtración selectiva. El nuevo aislado posee tan solo un 92% de similitud con *P. propionicigenes*, por lo que podría suponer un nuevo género dentro del *phylum Bacteroidetes*. Las secuencias cercanas al nuevo aislado han sido obtenidas en ambientes ácidos de minas o con alta concentración de metales pesados, lo que sugiere la naturaleza tolerante del aislado ante tales condiciones extremas.

Los productos de fermentación como el propionato y el butirato pueden ser oxidados a acetato, CO_2 y H_2. Esta oxidación es llevada a cabo por un grupo denominado "organismos acetógenos productores obligados de hidrógeno (OHPA)", mediante un proceso conocido como **acetogénesis**. Aunque la energía de este proceso es muy baja, su acoplamiento con metanógenas consumidoras de hidrógeno (que reducen la presión parcial de hidrógeno) la convierte en favorable. Esta relación se denomina sintrófica y la llevan a cabo bacterias del género *Syntrophobacter*. En los sedimentos del río Tinto, *Syntrophobacter* llega a representar el 37% del total en ciertos estratos aunque no está claro si actúa como bacteria sintrófica o BSR. También se han obtenido secuencias pertenecientes a *Oxobacter* spp., anaerobios obligados acetogénicos. La única especie, *Oxobacter pfennigii*, es un anaerobio esporulante que cataliza la transformación de piruvato a acetato y CO_2 (56).

El acetato e hidrógeno producido tras los procesos anteriores son típicos substratos de la **metanogénesis.** Aunque se considera una actividad anaerobia que se inhibe a bajos pHs, diversos estudios han detectado metanogénesis en condiciones ácidas incluso a pHs tan bajos como 4,5 (11, 24, 27, 33, 54, 107). Entre ellos, por su proximidad con este trabajo, destaca un estudio realizado usando sedimentos de la presa *JL* y *GAL* como inóculos (86).

Esto suscitó cuestiones sobre su presencia y extensión en un ecosistema tan ácido como el río Tinto, por lo que se realizó un *screening* con sedimentos de 8 puntos distribuidos a lo largo del río con 6 diferentes fuentes de carbono. Mediante la medida de la producción de metano, se constató la ubicuidad de las metanógenas en dicho ecosistema y su actividad en cultivos de enriquecimientos con pH alrededor de 5. La secuenciación del DNA de dichos enriquecimientos culminó con la identificación de arqueas como *Methanobrevibacter arboriphilus*, *Methanosarcina barkerii* o *Methanosaeta thermophile*. Además, por DGGE directa de DNA ambiental de un estrato reductor de los sedimentos de la presa *JL*, se ha obtenido una secuencia de arquea metanógena perteneciente a *Methanosaeta concilii* (JQ815591) coincidiendo con el estudio previo (86). *M. concilii,* que parece ser la arquea predominante en condiciones naturales, es una arquea metanogénica consumidora de acetato. El acetato, que ha sido medido en numerosas ocasiones en el agua intersticial de los sedimentos en concentraciones hasta 79 mg L^{-1}, es un producto de diversas fermentaciones y de oxidaciones parciales llevadas a cabo por BSR, metabolismos ambos presentes y abundantes en los sedimentos, por lo que la abundancia natural del substrato de *M. concilii* podría favorecer su prevalencia frente a otras metanógenas. Posiblemente las arqueas no jueguen un papel cuantitativamente importante en la degradación anaerobia de la material orgánica en el río Tinto, pero su presencia y actividad en sedimentos tan adversos tiene importantes connotaciones ecológicas –al ampliar el rango de tolerancia de dichas arqueas- y astrobiológicas –las detecciones de metano en la atmósfera de Marte podrían tener un origen biológico.

MODELO GEOMICROBIOLÓGICO DE LOS SEDIMENTOS DEL RÍO TINTO.

En esta tesis se ha abordado el estudio de los sedimentos del río Tinto combinando trabajos de ecología molecular microbiana y de microbiología tradicional e interpretándolos en función de los datos físico-químicos de los sedimentos. La integración de esta información biogeoquímica deriva en un modelo geomicrobiológico que integra los distintos ciclos de los elementos que operan en los sedimentos del río Tinto (Fe, N, C, y S) así como sus interacciones (Fig. 3).

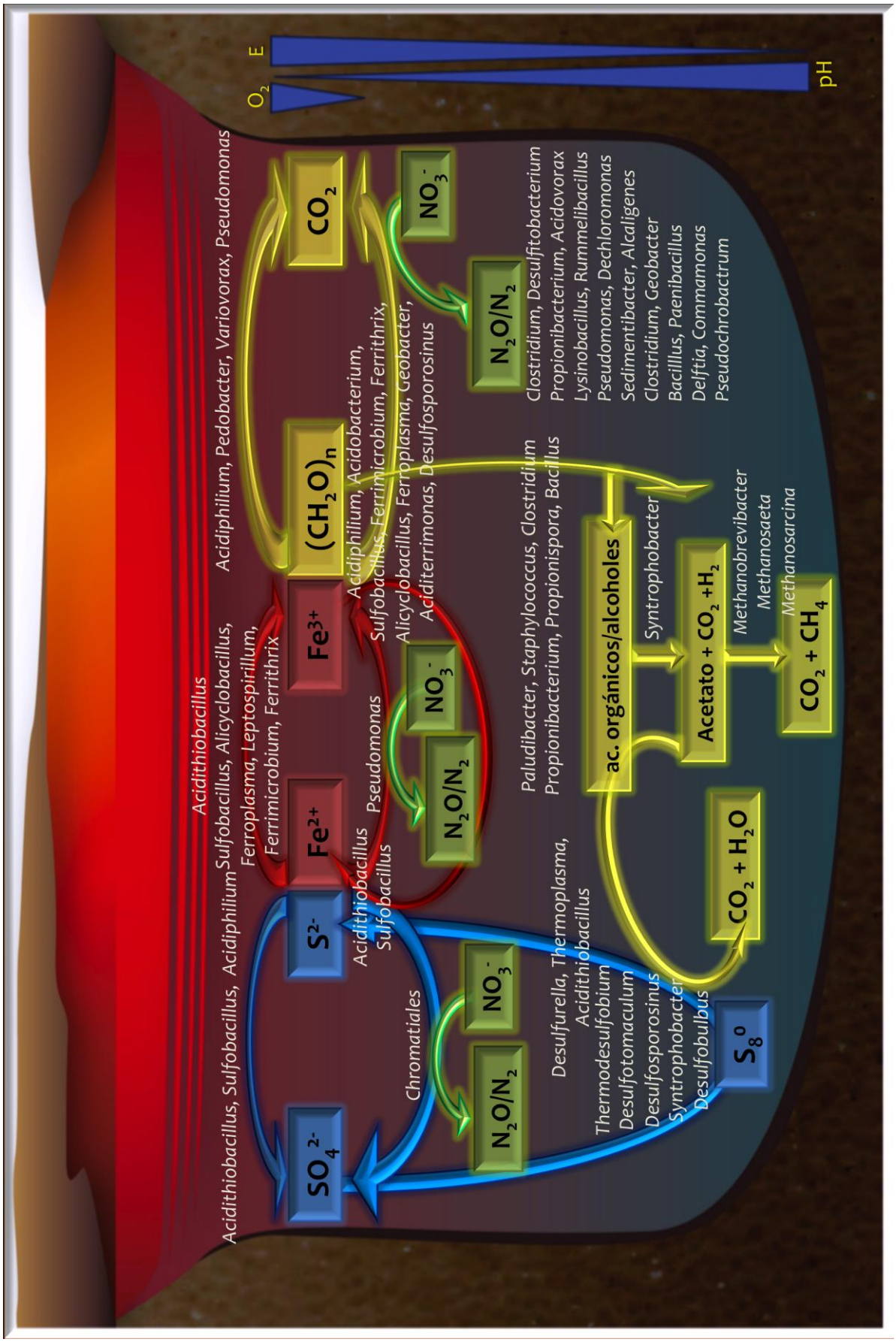

Figura 3: Modelo geomicrobiológico de los sedimentos del río Tinto. Los proceso implicados se señalan con flechas azules (ciclo del azufre), rojas (ciclo del hierro), amarillas (ciclo del carbono) y verdes (ciclo del nitrógeno).

A diferencia de los estudios previos relativos a la columna de agua del río, la ecología microbiana de los sedimentos no está basada fundamentalmente en el ciclo del hierro. De hecho, parece que el ciclo del azufre y la utilización de compuestos orgánicos dominan este subsistema del Tinto, con diversas interacciones con el ciclo del nitrógeno y hierro. Partiendo de los substratos que proporciona el lecho de la Faja Píritica Ibérica, principalmente pirita (FeS_2), los compuestos reducidos de hierro y azufre serán el substrato disponible para los quimiolitótrofos, siendo la base para el desarrollo de una compleja comunidad.

El hierro en su forma reducida, Fe(II), sería la principal fuente de energía en condiciones aerobias o microaerófilas para bacterias como *At. ferrooxidans, Sulfobacillus* spp., *Alicyclobacillus* spp., *Ferrimicrobium* spp y arqueas como *Ferroplasma* spp; y en condiciones de anaerobias para *Pseudomonas* spp. o *Alicyclobacillus* spp. acoplado a la reducción de nitratos. Los sistemas ácidos son ricos en hierro debido a su alta solubilidad a bajo pH, excediendo las concentraciones de Fe(III) a las de oxígeno en dos órdenes de magnitud, de modo que el Fe(III) puede ser usado como aceptor de electrones por multitud de microorganismos (17, 22). Así, el Fe(III) puede ser reducido acoplado a la oxidación de materia orgánica por heterótrofos acidófilos como *A. capsulatum* , *Acidiphilium* spp., *Sulfobacillus* spp., *Ferrimicrobium* spp., etc. o acoplado a la oxidación de azufre por *At. ferrooxidans* y *Sulfobacillus* spp. Estos procesos implican la regeneración del ferroso completándose el ciclo del hierro con los organismos identificados en los sedimentos del río Tinto.

El segundo grupo de metabolitos cruciales en este sistema son los compuestos del azufre. El sulfuro puede ser oxidado a sulfato en condiciones aerobias o anaerobias (acoplado a la reducción de hierro mencionada) por *At. ferrooxidans* y *Sulfobacillus* spp. El sulfato puede ser reducido desasimilativamente por bacterias como *Desulfosporosinus* spp., *Thermodesulfobium* spp., *Syntrophobacter* spp., *Desulfotomaculum* spp., etc. Un metabolito intermedio como el azufre elemental, también puede ser reducido completamente a sulfuro por BSR como *Desulfurella, At. ferrooxidans* o arqueas como *Thermoplasma* spp. La presencia y actividad demostrada de SBR en el sistema evidencia la existencia de la sulfato-reducción en condiciones ácidas, cerrando el ciclo del azufre en sedimentos extremos como los del río Tinto.

Respecto al ciclo de nitrógeno, ciertos organismos de los géneros *Leptospirillum*, *Ideonella* o *Anaerobacter* podrían fijar nitrógeno atmosférico. Además, diversos organismos - tales como miembros de los géneros: *Alcaligenes*, *Pseudochrobactrum*, *Pseudomonas*, *Desulfitobacterium*, *Delftia*, etc.- llevan a cabo la desnitrificación acoplada a la oxidación de azufre, de hierro ferroso o de materia orgánica

Y por último, el ciclo del carbono, con el que interactúan un número considerable de los organismos implicados en los otros tres ciclos. Entre los organismos heterótrofos encontramos metabolismos tanto respiratorios (aerobios y anaerobios) como fermentadores. La oxidación de la materia orgánica podría llevarse a cabo en condiciones aerobias o microaerobias por *Acidiphilium*, *Pedobacter*, *Variovorax* y una gran variedad de respiradores, o anaerobias acoplada a la reducción del hierro, del sulfato o del nitrato antes mencionados. Adicionalmente, se dan procesos fermentativos llevados a cabo por representantes de géneros como *Clostridium, Bacillus, Propionibacterium, Veillonella, Paludibacter, Staphylococcus,* etc. Estas fermentaciones crean una compleja mezcla de intermediarios como ácidos grasos volátiles, alcoholes e hidrógeno. Muchos de estos productos pueden ser oxidados hasta acetato por BSR o por bacterias sintróficas como *Syntrophobacter* spp., asociado, en este

último caso, a la producción de hidrógeno. Estos compuestos son los sustratos principales de otros organismos, tanto respiradores aerobios o anaerobios, como de las arqueas metanogénicas *Methanosaeta*, *Methanosarcina* o *Methanobrevibacter*.

En estudios previos en perforaciones del subsuelo en el entorno de río Tinto (5), se había detectado la presencia de gases como H_2, NO_2 y CH_4 en el fluido subterráneo extraído, proponiéndose el origen químico del H_2 derivado de la interacción roca-agua. La identificación de organismos productores de hidrógeno como *Syntrophobacter* spp. y *Clostridium* spp., productores de metano como *Methanosaeta*, *Methanosarcina* y *Methanobrevibacter* y diversos desnitrificantes sugiere la posibilidad de producción biológica de estos gases.

En conclusión, la puesta en común de los datos recopilados en la presente tesis permite una compresión profunda de los distintos ciclos geoquímicos que operan en los sedimentos del río Tinto, así como sus complejas interacciones. Además, las actividades inferidas mediante técnicas de ecología molecular han sido demostradas con técnicas de microbiología clásica y medidas cuantitativas, validando el modelo propuesto.

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CONCLUSIONES GENERALES

1. Los datos de clonaje y DGGE muestran que la biodiversidad procariótica en los sedimentos, especialmente la diversidad bacteriana, es significativamente mayor que la encontrada en estudios previos en la columna de agua. Frente a la uniformidad físico-química en dicha columna, la protección que confiere la estructura de los sedimentos permite el desarrollo de diferentes nichos ecológicos que albergan consorcios microbianos más diversos.

2. La densidad de microorganismos en los sedimentos del río Tinto varía sustancialmente entre distintos puntos de muestreo, moviéndose en el rango 10^7 - 10^8 células por gramo de peso húmedo de sedimento. Además también varía con la profundidad, disminuyendo hasta 10 veces su valor a lo largo de los primeros 50 cm de profundidad.

3. Los datos de CARD-FISH muestran que los microorganismos pertenecientes al dominio *Bacteria* (>98%) dominan sobre *Archaea* (<2%), no habiéndose detectado organismos activos pertenecientes al dominio *Eukarya*.

4. Se ha diseñado una sonda específica para el género *Desulfurella* (DSU655). Dicha sonda ha permitido cuantificar - mediante CARD-FISH - la abundancia de *Desulfurella* spp. en los sedimentos del río Tinto.

5. En los sedimentos ácidos del río aparecen ciertas actividades anaerobias que tienen lugar preferentemente a pHs neutros, tales como metanogénesis, sulfato-reducción, producción de hidrógeno y desnitrificación, lo que evidencia el amplio rango de tolerancia de los microorganismos a las condiciones ambientales. Estas actividades confirman la producción biológica de gases como H_2, CH_4 y N_2O en el subsuelo del río Tinto.

6. Mediante técnicas moleculares, se han identificado microorganismos relacionados con el ciclo del hierro (*At. ferrooxidans*, *Sulfobacillus* spp., *Ferroplasma* spp., etc.), azufre (*Desulfurella* spp., *Desulfosporosinus* spp., *Thermodesulfobium* spp., etc.), carbono (*Acidiphilium* spp., *Bacillus* spp., *Clostridium* spp., *Acidobacterium* spp., etc.) y nitrógeno (*Alcaligenes* spp., *Pseudochrobactrum* spp., etc.), y su distribución se ha correlacionado con los parámetros fisicoquímicos de los sedimentos.

7. La distribución de los organismos en los sedimentos está íntimamente relacionada con las características físico-químicas de los mismos (pH, potencial redox, sulfato y concentraciones de metales pesados); las comunidades varían entre distintos puntos de muestreo e incluso entre distintas profundidades en el mismo punto. Cuando los parámetros son más próximos a los de la columna de agua (pH 2,5 y +400 mV), los organismos más abundantes corresponden a reductores de hierro (*Acidithiobacillus*, *Acidiphilium* , etc.) mientras que cuando aparecen mayores pHs (4,2-6,2) y potenciales redox más reductores (50, -246 mV), los organismos relacionados con reducción de sulfato parecen predominar (*Desulfosporosinus*, *Desulfurella*, *Syntrophobacter*, etc.).

8. Los datos obtenidos mediante cultivos de enriquecimiento vinculan la sulfato-reducción con representantes de los géneros *Desulfotomaculum*, *Thermodesulfobium*, *Desulfitobacterium* y especialmente, *Desulfosporosinus*; la desnitrificación con diversos organismos pertenecientes a géneros como *Paenibacillus*, *Bacillus*, *Sedimentibacter*, *Lysinobacillus*, *Delftia*, *Alcaligenes*, *Clostridium* y *Desulfitobacterium*; las especies del

género *Clostridium* parecen ser los principales productores de hidrógeno en este ecosistema y por último, aunque parece tener escasa relevancia ecológica, la metanogénesis aparece vinculada a la presencia de arqueas de los géneros *Methanosarcina*, Methanosaeta y *Methanobrevibacter*.

9. Se han aislado bacterias productoras de hidrógeno, sulfato-reductoras y fermentadoras. Entre las sulfato-reductoras, dos cepas son próximas (96%) a *Desulfosporosinus acidophilus,* una a *Desulfosporosinus orientis (96%),* y otra al grupo *Desulfitobacterium/Desulfosporosinus* (93%), por lo que puede tratarse de dos nuevas especies y un nuevo genero dentro de la familia *Peptococcaceae* (*Firmicutes*). Un aislado con metabolismo fermentativo presenta un porcentaje de semejanza de tan sólo un 92% con *Paludibacter propionicigenes*, por lo que podría suponer un nuevo género dentro del phylum *Bacteroidetes*.

10. Nuestros resultados sugieren que se produce una atenuación natural de las características extremas de las aguas ácidas de mina (pH ácido y alta concentración de metales en disolución) mediada por bacterias sulfatorreductoras acidófilas, con la consecuente precipitación de metales en forma de sulfuros. Este proceso pudo ser usado para biorremediación: el tratamiento conjunto de aguas ácidas con aguas residuales urbanas produjo un efluente de calidad con mínimo coste económico y ambiental.

11. La identificación de diversos microorganismos, el examen detallado de sus metabolismos correspondientes, su cuantificación y su cultivo han permitido desarrollar y validar un modelo de funcionamiento de los ciclos del azufre, hierro, carbón y nitrógeno en los sedimentos del río Tinto en relación a la ecología microbiana de los mismos.

GENERAL CONCLUSIONS

1. Cloning and DGGE data show that prokaryotic diversity in the sediments, especially bacterial diversity, is significantly greater than that found in previous studies in the water column. In contrast to the homogeneity of the water column, the protection that the sediment's structure confers, allows for the development of diverse ecological niches that host more diverse microbial consortia.

2. The density of microorganisms in sediments of the Tinto river varies substantially between different sampling points, ranging from 10^7 to 10^8 cells per gram of wet weight of sediment. In addition, the density also varies with depth, decreasing up to 10 fold over 50 cm of depth.

3. CARD-FISH data show that microorganisms belonging to *Bacteria* (>98%) dominated over *Archaea* (<2%) while no active cells of the domain *Eukarya* were detected.

4. A specific probe for the genus *Desulfurella* (DSU655) has been designed. This probe has allowed for the quantification –by CARD-FISH- of the abundance of Desulfurella spp. in the Tinto river sediments.

5. In the acidic sediments of Tinto river, certain anaerobic activities that take place preferentially at neutral pH, such as methanogenesis, sulfate-reduction, hydrogen-production and denitrification, occur, which shows the great tolerance of microorganisms to extreme conditions. The detected and quantified activities confirm the bioproduction of H_2, CH_4 and N_2O in the subsurface of Tinto river.

6. Microorganisms involved in the iron (*Acidithiobacillus ferrooxidans*, *Sulfobacillus* spp., etc.), sulfur (*Desulfurella* spp., *Desulfosporosinus* spp., *Thermodesulfobium* spp., etc.), carbon (*Acidiphilium* spp., *Bacillus* spp., *Clostridium* spp., *Acidobacterium* spp., etc.) and nitrogen (*Alcaligenes* spp., *Pseudochrobactrum* spp., etc.) cycles were identified. The detection of these microorganisms, a detailed examination of their metabolism, and their quantification and cultivation have allowed for the compilation of a model of the iron, sulfur, nitrogen and carbon cycles and their relation to microbial ecology in extreme acidic sediments.

7. The distribution of organisms in the sediments is closely related to their physico-chemical characteristics (pH, redox potential, sulfate and concentrations of heavy metals); the communities vary between different sampling sites and even between different depths at the same site. When the parameters are closest to those of the water column (pH 2.5 and + 400 mV), the most abundant organisms are iron reducers (*Acidithiobacillus, Acidiphilium* , etc.) while when higher pHs (4.2-6.2) and more reducing redox potentials (50, - 210 mV) appear, sulfate-related organisms seem to predominate (*Desulfosporosinus, Desulfurella* and *Syntrophobacter*).

8. Data from enrichments suggests that the sulfate-reduction is linked to representatives from the *Desulfotomaculum*, *Thermodesulfobium*, *Desulfitobacterium* and, especially, *Desulfosporosinus* genera. Denitrifying organisms showed a broad diversity, scattered among the genera *Paenibacillus*, *Bacillus*, *Sedimentibacter*, *Lysinobacillus*, *Delftia*, *Alcaligenes*, *Clostridium* and *Desulfitobacterium*. Hydrogen-producing enrichments were dominated by *Clostridium* spp. Methanogenesis, although of scarce ecological

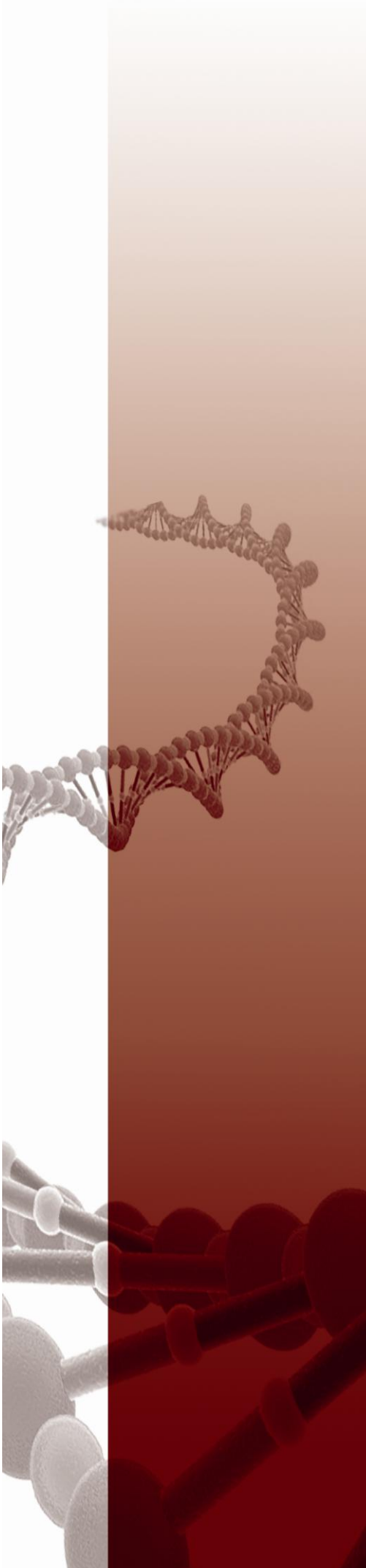
relevance, appeared to be related with the archaeal genera *Methanosarcina*, *Methanosaeta* and *Methanobrevibacter*.

9. Strains of hydrogen-producers, sulfate-reducers and fermenters, have been isolated from Tinto river sediments. Among the sulfate-reducers, two strains share 96% of similarity to *Desulfosporosinus acidophilus*, another 96% to *Desulfosporosinus orientis* and another one has just 93% 16S rRNA gene sequence similarity with the clade *Desulfitobacterium/Desulfosporosinus;* the might represent two new species and a new genus in the family *Peptococcaceae* (*Firmicutes*) Another isolate with fermentative metabolism presents a percentage of similarity of just 92% to the closest phylogenetic isolate: *Paludibacter propionicigenes*, so it might represent a new genus in the *Bacteroidetes* phylum.

10. Our results suggest that a natural attenuation of ARD characteristics (low pH and high heavy metal content) is biologically driven by sulfate-reducers and the consequent precipitation of metals as sulfides. The application of this biotechnological potential was possible coupling the bioremediation of Acid Mine Drainage waters with domestic waste treatment producing a quality effluent with a minimal economic and ecological cost.

11. The identification of different microorganisms in this study, a detailed examination of their corresponding metabolism, their quantification and their cultivation have allowed us to compile and validate a model of the iron, sulfur, nitrogen and carbon cycles and their relation to microbial ecology in Tinto river sediments.

ANEXO: Trabajo en proceso



Caracterización del aislado fermentador

Uno de los estudios de la presente tesis (capítulo 5) se obtuvo un co-cultivo de una fermentadora y una BSR.

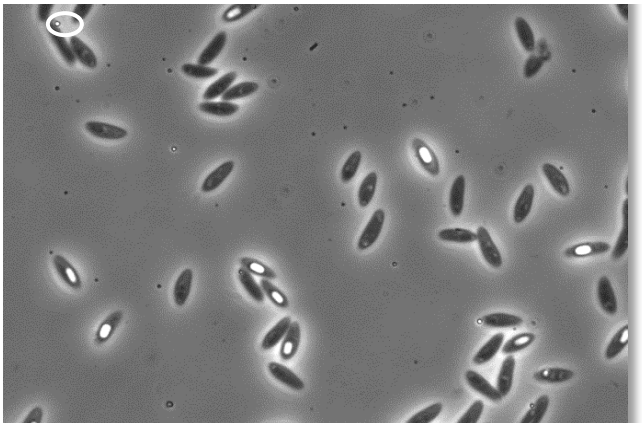


Figura 6: Imagen del cocultivo tomada mediante microscopía electrónica. Puede apreciarse la diferencia de tamaños entre la BSR (,4 µmx1µm), resaltada en amarillo y la fermentadora (1 µmx0,3µm), resaltada en blanco

Tras filtrar el co-cultivo a través de un filtro con un diámetro de poro de 0,45 µm, las bacterias SRB quedaron retenidas mientras que las fermentadoras pasaron al filtrado. Tras 3 diluciones seriadas y 3 transferencias sucesivas en placas de agar, el estudio culminó con la obtención de un aislado de un organismo fermentador con 92% de similitud con el aislado más próximo que corresponde a *Paludibacter propionicigenes.* Este aislado podría representar un nuevo género dentro del *phylum Bacteroidetes*, por lo que se ha procedido a su caracterización.

Análisis de pH, temperatura, salinidad y substratos:

Para conocer los rangos y los óptimos de crecimiento del aislado para distintos parámetros, se creció el aislado bajo los siguientes parámetros: pH (3-13), temperatura (4-80 °C), salinidad (0-3 %) y 10 mM de concentración de sustratos (almidón soluble , sacarosa, lactosa, xilosa, arabinosa, celobiosa, manosa, galactosa, glucosa, maltosa, L-sorbosa, acetato, arsenato, piruvato, butirato, malato, propionato, lactato, benzoato, succinato, citrato, nitrato, metanol, etanol, 2-propanol, manitol, glicerol, SO_4^{-2}, Fe^{3+}). Los ensayos se realizaron por triplicado en el medio anaerobio anteriormente descrito en el capítulo 5 suplementado con 10 mM de extracto de levadura (excepto en los ensayos de substratos). Se inoculó 1 mL de cultivo en botellas anaerobias de 50 mL, dejando transcurrir 15 días.

El rango de temperatura de crecimiento del aislado es de 22-38,5 °C (Figura 1). La temperatura óptima fue de 37 °C observándose una disminución de pH paralela al aumento de DO, típico en los cultivos fermentadores debido a la liberación de ácidos.

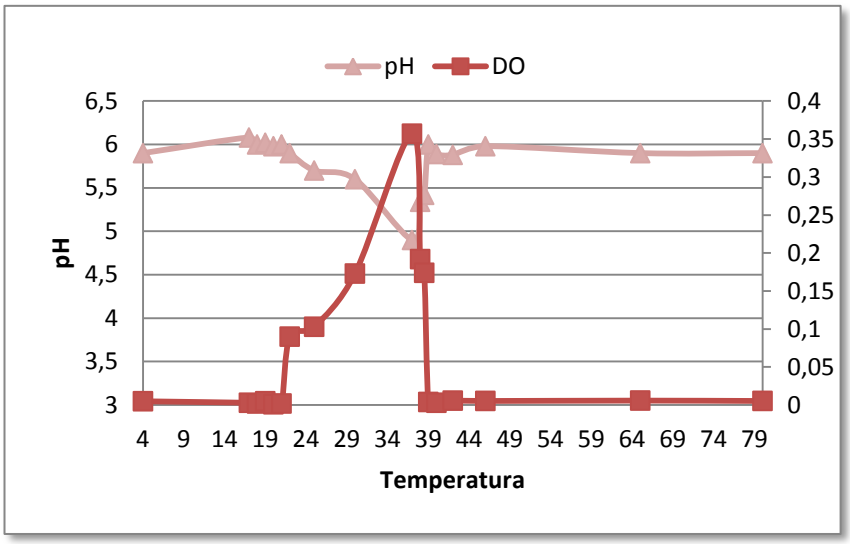


Figura 1. Optimización de la temperatura. Se puede apreciar un valor máximo a los 37°C, en cuanto al rango de crecimiento, se sitúa entre valores de 22-38,5 °C.

Por lo que se refiere al pH, el óptimo se sitúa en un valor de 5,75, con un rango de crecimiento entre 4,9-7 (Figura 2).

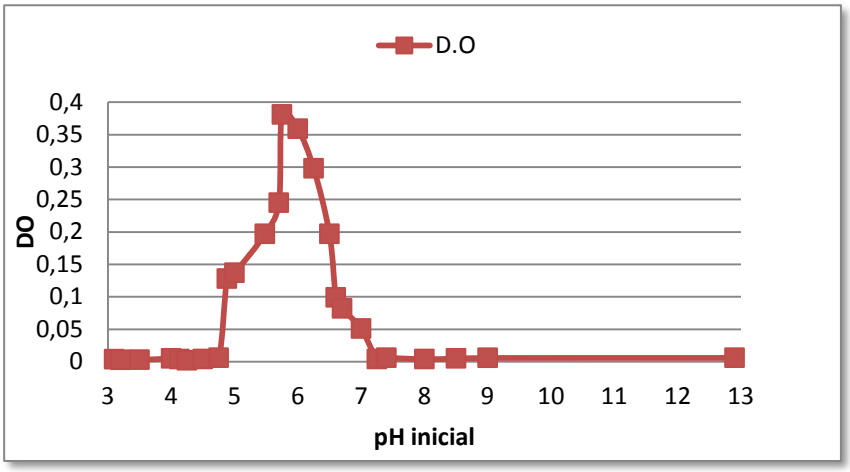


Figura 2. Optimización del pH. Se muestra un óptimo valor cuando el cultivo alcanza un pH de 5,75, permitiendo el crecimiento en un rango de 4,9-7.

Respecto al rango de salinidad en el que se desarrolla el cultivo, éste tolera valores entre 0-1,6% de NaCl (Figura 3).

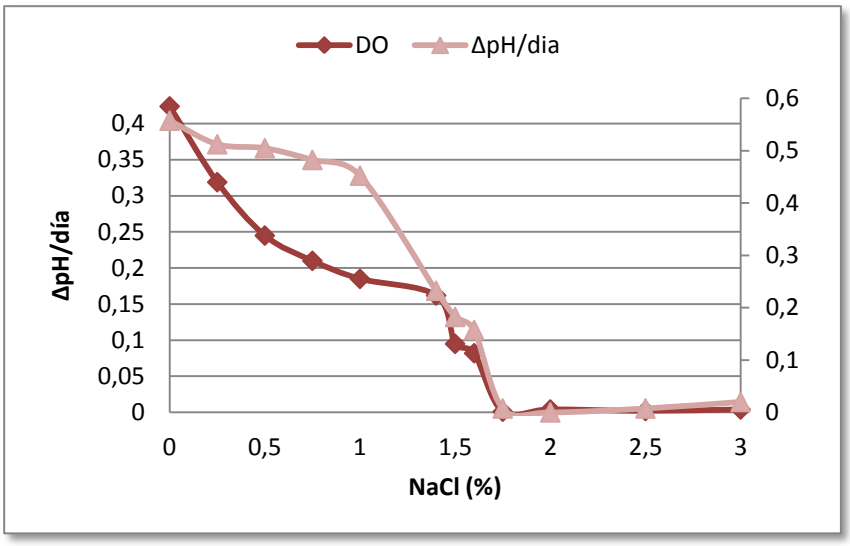


Figura 3. Optimización de la salinidad.

Respecto a la utilización de substratos, mostró crecimiento con almidón soluble, maltosa, glucosa, fructosa, xilosa, arabinosa, celobiosa, manosa, galactosa. Como productos de fermentación se midieron acetato y propionato en todos los casos en una relación media de 1,8:1 aunque variaba en función del substrato (Fig. 4).

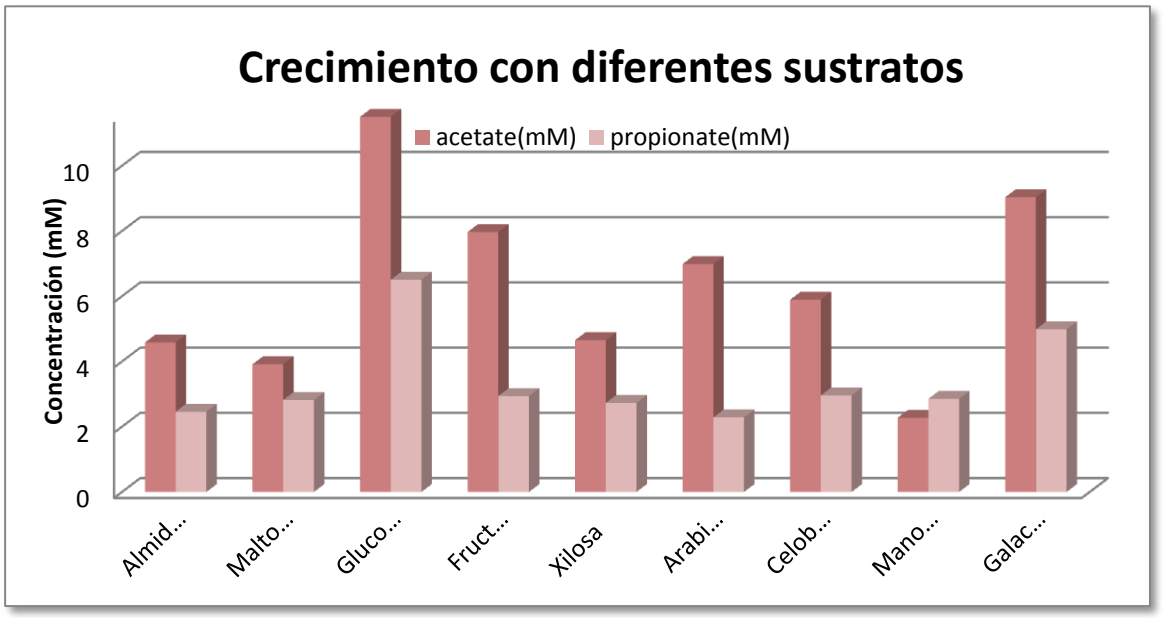


Figura 4. Producción de ácidos en función del substrato utilizado.

Los ensayos de utilización de substratos se complementaron con el test API20. Resultó positivo para la reacción/enzima β-galactosidasa, arginina dihidrolasa, lisina descarboxilasa, ornitina, ureasa y para las fermentaciones de glucosa, ramnosa, melibiosa, arabinosa.

Con objeto de probar la pureza del aislado, los cultivos crecidos con distintos sustratos se sometieron a DGGE. El patrón de bandas resulto ser siempre el mismo, confirmando que se trata de un cultivo homogéneo (Figura 5).

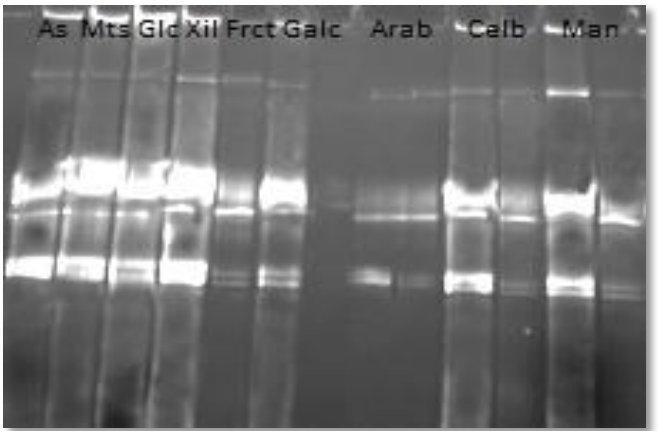


Figura 5. Patrones de bandas obtenidos mediante DGGE. As: almidónsoluble, Mts: maltosa, Frct: fructosa, Gluc: glucosa, Xil: xilosa, Arab: arabinosa, Celb: celobiosa, Man: manosa, Galc: galactosa

Por lo que se refiere a sus características bioquímicas, morfológicas y desarrollo, el organismo aislado es estrictamente anaerobio, Gram negativo, no formador de esporas y con un aspecto de varillas cortas de 1,1 micras de longitud por 0,4 micras de diámetro.

Presenta resultados negativos para la peroxidasa, catalasa, reducción de nitrato y de hierro. En condiciones óptimas y a una concentración inicial de sustrato de 10mM, el cultivo mostraba crecimiento a las 72 horas y alcanzaba valores de 2,8 x10 ^{9} cel mL^{-1} al cabo de una semana.

Conclusiones: El aislado muestra crecimiento en un rango de temperaturas entre 22-38,5 ºC con óptimo a 37ºC; un rango de pH entre 4,9-7 con valor óptimo en 5,75; y un rango de salinidad de 0-1,6%. El aislado es anaerobio estricto, con metabolismo fermentador capaz de utilizar para su crecimiento almidón soluble, maltosa, glucosa, fructosa, xilosa, arabinosa, celobiosa, manosa, galactosa, aunque presentando un mayor crecimiento con glucosa y galactosa. Como principales productos de su metabolismo se generan, con independencia del sustrato utilizado, acetato y propionato, lo que apunta a una fermentación propiónica y/o ácido mixta.