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Bioleaching of pyritic coal wastes: bioprospecting and efficiency of selected consortia

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ABSTRACT

Pyrite-bearing coal wastes are responsible of the formation of acid mine drainage (AMD), and their management to mitigate environmental impacts is a challenge to the coal mine industry in Europe and worldwide. The European CEReS project sought to develop a generic co-processing strategy to reuse and recycle coal wastes, based on removal of AMD generating potential through bioleaching. Chemolitoautotrophic iron- and sulfur-oxidizing microbial consortia were enriched from a Polish coal waste at 30 °C and 48 °C, but not 42 °C. Pyrite leaching yield, determined from bioleaching tests in 2-L stirred bioreactors, was best with the 48 °C endogenous consortium (80%), then the 42 °C exogenous BRGM-KCC consortium (71%), and finally the 30 °C endogenous consortium (50%). 16S rRNA gene-targeted metagenomics from five surface locations on the dump waste revealed a microbial community adapted to the site context, composed of iron- and/or sulfur-oxidizing genera thriving in low pH and metal rich environments and involved in AMD generation. All together, the results confirmed the predisposition of the pyritic coal waste to bioleaching and the potential of endogenous microorganisms for efficient bioleaching at 48 °C. The good leaching yields open the perspective to optimize further and scale-up the bioleaching process.

1. Introduction

The coal mining industry generates large volumes of coal production wastes worldwide. In Europe, 2.4 gigatons of coal production wastes and tailings are stockpiled over several countries. Coal is formed in reducing environments, and contains a variety of minerals, often including sulfide minerals among which pyrite (FeS_2) is by far the most common [1]. The sulfide minerals are removed during coal beneficiation processes, and report to production wastes. These minerals are prone to oxidation and can generate acid mine drainage (AMD). AMD from underground and surface mines, waste dumps and tailing dams is one of the oldest and most consistent industrial problems facing coal mining regions in Europe and elsewhere [2–4], and environmental damage due to AMD from sulfidic coal production wastes is a significant challenge to the European coal industry.

AMD is formed when iron sulfide minerals react with oxygen and water to form a highly acidic solution rich in sulfate and dissolved iron and other metals. Naturally occurring iron- and sulfur-oxidising acidophilic microorganisms greatly accelerate the formation of AMD [5]. Current best practice is to prevent the formation of AMD through capping of sulfidic mine wastes and/or other containment strategies, or to treat it post-genesis (where complete prevention is not possible) [6,7]. However, neither of these methods address the source of the problem: the sulfide component. As a result, capping materials require regular monitoring, and treatment options engage significant financial commitments for potentially hundreds of years. Many former mines and waste dumps are environmental ticking time bombs.

Reuse and recycling of mine wastes are among the favoured options in mine waste management strategies [8]. Common options for the reuse of coal production wastes is in civil engineering projects or backfill and landscaping of the mine, however, the instability of sulfide-bearing coal wastes limits the possibilities to

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reuse or recycle them. Indeed, significant environmental and geotechnical stability issues can be seen where sulfide-bearing wastes have been used in this way (*e.g.* Buków flood polder, ground levelling and backfilling) [9,10].

In the case of sulfide-bearing wastes from metal mining, there may be sufficient residual valuable metal content to apply biomining technologies (biohydrometallurgy) to reprocess them. Biomining harnesses the activity of the microorganisms which normally catalyse the formation of AMD dissolve the metal-bearing sulfide mineral, producing a metal-rich pregnant leach solution (PLS; from which the metals can be refined) and an environmentally benign solid waste devoid of acid-generating potential [11,12]. This was applied at the former Kasese Copper mine in Uganda to recover cobalt from pyritic mine tailings [13]. The cobalt was sold at a profit while removing the sulfide content of the tailings alleviated the environmental damage from the site. This is a win–win situation, generating a net profit while permanently removing the potential to generate AMD from the mine tailings.

This depollution approach is more of a challenge where the wastes do not contain any valuable metals, such as is the case with pyritic coal waste; there is insufficient value to cover the processing costs. The CEReS concept seeks to address this by taking a co-processing approach (http://ceres.biohydromet.net/). The process uses bioleaching to remove the AMD-generating potential from the coal wastes. The solid residues can be used in geotechnical applications, while the ferric iron-rich acidic solution is used as a cheap leaching solution to recover valuable metals from processed electronic wastes (see [14] for a detailed explanation of the CEReS concept). The first step in proving the CEReS concept was the RFCS-funded EU project "CEReS" which sought to demonstrate the technical feasibility of the individual process units at lab–pilot scale before integrating them in a process simulator [14]. The project used Poland as a case study country (the largest hard coal producer in Europe with 31 coal mines producing about 74 Mt hard coal *pa* and 600 million tons of wastes currently stockpiled in the country [9,15]), and involved an active coal mine producing sulfidic production wastes.

This paper details work done on the first step of the co-processing strategy of the CEReS project: the removal of the pyritic sulfur contained in a coal production waste through bioleaching.

Commercial stirred tank bioleaching operations typically operate at 40–45 °C at pulp densities of 20% and overall retention times of around 5 days [*e.g.* 15, 16]. While increasing temperature may increase the rate of reaction, it also decreases the solubility of ferric iron and therefore may affect the final yield (*e.g.* by co-precipitation with target metals). Running at cooler temperatures may improve final yield, but will increase the retention time; potentially making a process uneconomic. However, as the CEReS approach does not depend on the dissolution of a valuable metal it may be that it can be run at a lower temperatures if this produces more stable solid residues (less un-oxidised pyrite) and better quality leaching solution (higher ferric iron concentration) for use in the leaching of processed e-wastes. On the other hand, the oxidation of pyrite is an exothermic process, and much of the capital and operating expenditure (CAPEX and OPEX) of commercial plants is linked to the cooling required to maintain the reaction vessels at 40–45 °C [16]. Therefore, running at hotter temperatures may help reduce processing costs, but this will be a trade-off with the potential benefits of running at cooler temperatures (~30 °C).

The biodesulfurisation of coal (oxidation of sulfides and/or organic sulfur in order to produce a saleable coal product) is well documented, from laboratory-scale experiments to large-scale pilot operations (*e.g.* semi-commercial stirred-tanks [17], 6 ton heap leaching pilot [18], or in a full-scale packed-bed bioreactor [19]). However, the application of bioleaching as a method of depollution of coal production wastes has not been studied before.

The microbial communities involved in the bioleaching of metal ores are exposed to very high soluble metal contents (*e.g.* [20]), which will not be the case in the bioleaching of pyritic coal wastes. As such, the ecological drivers in these systems, as well as the waste dumps from which these communities originate, may be quite different. This may affect the types of communities that can be enriched and selected for bioleaching.

The objectives of this study were: (1) to obtain new microbial acidophilic consortia endogenous to the waste dump at temperatures ranging from mesophilic to moderately thermophilic (30 °C, 42 °C and 48 °C), (2) to study their bioleaching efficiency, benchmarked to that of an existing consortium used in a commercial bioleaching plant, and (3) to examine the diversity of microbial communities inhabiting the waste dump though 16S rRNA gene targeted metagenomics.

2. Materials and methods

2.1. Coal production waste materials

Eleven samples were collected in 2016 for microbial enrichments from a heap of pyrite-bearing coal production waste on site in the Janina coal mine (Libiąż, Poland). The heap contains mineral processing wastes from the beneficiation and concentration of the coal. Due to the acidogenic potential of this material, the heap is associated with the formation of AMD. A further five samples were collected from the dump in 2018 for microbial community metagenomic analysis.

For bioleaching tests in reactors, a representative 386 kg coal mining tailings sample was collected downstream of a spiral concentrator on site in 2016, by Tauron Wydobycie Spolka Akcyjna (TW) and Glowny Instytut Gornictwa (GIG); it was screened (using 20 mm and 5 mm sieves), homogenised and subdivided (with riffle splitter) to generate representative subsamples of 25 kg, which were further splitted for the reactor tests. The material used for the bioleaching tests contained: 5.7% Fe, 6.6% S (98.5% as sulfides), 0.9% K, 1.6 ppm N, 15.9% TOC (Fe and K were determined by ICP-AES after a total sample digestion with a sodium peroxide sinter; total S was determined by combustion; N is the sum of Kjeldahl N and NO_3 determined by ionic chromatography after lixiviation; TOC was determined with a Horiba EMIA 820 V analyser after a pre-treatment with HCl to eliminate mineral carbon).

2.2. Cultivation and taxonomic characterization of bioleaching consortia from the pyritic coal wastes

In order to obtain bioleaching cultures endogenous to the pyritic coal wastes from the site, microbial consortia were enriched from the 11 waste dump samples in minimal salts medium (MSM; 100 mL with 3% w/v solids) in aerobic agitated (150 rpm) shake flasks at three temperatures: 30 °C, 42 °C and 48 °C, giving rise to 33 enrichments in total. The MSM contained (g L^{-1}): $(NH_4)_2SO_4$, 0.4; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.2, at an initial pH of 1.8 (adjusted with H_2SO_4). Growth was assessed by monitoring the generation of acid (decreasing pH), production of soluble Fe^{3+} and growth of planktonic prokaryotic cells (microscopic cell counts). Fe speciation was determined spectrophotometrically by complexing Fe^{3+} with chloride and then reading for absorbance at 340 nm, as described in detail in [21]. The 11 cultures at each temperature were pooled to obtain three endogenous consortia: TW30 at 30 °C, TW48 at 48 °C and TW42 at 42 °C. Taxonomic characterization of the enriched consortia was assessed by cloning and sequencing of 16S rRNA genes. For molecular characterization of the consortia, 2 mL of

culture were centrifuged (10 min, 14,000 g), pellets were washed by re-suspension in 1 mL Tris buffer (100 mM, pH 8) to increase the pH around 7 and avoid DNA degradation during the extraction, and stored at −20 °C prior use. Microbial DNA was extracted from the frozen, washed pellets with the FastDNA Spin Kit for Soil and using the manufacturer's protocol (MP Biomedicals) with a FastPrep®-24 instrument at a speed of 5 $m.s^{-1}$ for 30 s. About 1.4 kb of the 16S rRNA gene was PCR amplified with primers 8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer 1492R (TACGGTTACCTTGTTACGAC-3′), and purified PCR products (NucleoSpin Extract II, Macherey–Nagel) were cloned using the TOPO-TA Cloning Kit for sequencing (Invitrogen). Insert sequencing from colonies carrying correct-length inserts was performed with vector primers T3 and T7 by the company Eurofins Genomics. Nucleotide sequences were manually verified (BioEdit software; [22]) and 28, 18 and 37 good-quality 16S rRNA gene consensus sequences were obtained for TW30, TW42 and TW48, respectively. Consensus sequences were compared by Blast (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to identify their relationships with 16S rRNA gene sequences of known strains.

2.3. Bioleaching efficiency of selected consortia in bioreactors

Tests were conducted in mechanically agitated and aerated batch stirred-tank bioreactors (STR) in order to test the bioleaching performances, in terms of kinetics and leaching yield of the pyrite contained in the coal production waste, of the following bioleaching consortia: the endogenous TW30 and TW48 consortia enriched form the coal waste, at their respective driving temperature of 30 °C and 48 °C, and the allochthonous BRGM-KCC consortium, already applied with high performances in several pilot and commercial bioleaching applications (*e.g.* [23–25]), at 42 °C. Prior to these tests, TW30, BRGM-KCC and TW48 consortia were subcultured several times at 30 °C, 42 °C and 48 °C, respectively, first in shake flasks at 3% solid concentration, then in STR at 5% and 10% solid concentration. This procedure enables the microorganisms to adapt to growing solid concentration and is detailed in [26]. The cultures obtained at the end of the procedure were used as inocula for the tests described below.

2.3.1. Experimental apparatus

The experimental setup is fully described in [24]. It included 2 L glass bioreactors with jackets where warm water is flowing to maintain a constant operating temperature. The temperature of the circulating water was regulated by a cryothermostat and thermocouples placed in the bioreactors. All the bioreactors were equipped with four baffles mounted 90° apart and extended down to the base of the vessel to optimise the mixing of pulp. The agitation was performed using a dual impeller system (axial/radial) consisting of a standard 6-blade Rushton turbine in combination with a 3-blade 45° axial flow impeller. The gas supply system was designed to accommodate air enriched with 1% CO_2 which was injected beneath the turbine at the bottom of the bioreactors *via* a stainless steel pipe. The impellers and the gas injection pipe were positioned in order to respect the standard dimensions and thus, to optimize gas mass transfer and mixing in the bioreactors. The top of the reactors was connected to a gas cooling system to prevent excessive evaporation.

2.3.2. Experimental procedure

The bioleaching tests were carried out in batch mode in series, and four successive runs were performed in the following conditions: run 1, named R2L1, at 5% (w/v) solid concentration and medium 0Km; run 2, named R2L2, at 10% (w/v) solid concentration and medium 0Km; run 3, named R2L3, again at 10% (w/v) solid concentration and medium 0Km; and run 4, named R2L4, at 10% (w/v) solid concentration and medium 0Cm. The 0Km nutrient medium [27] ("K" medium without iron, "m" indicating modification of the basal salts) is optimised for bacterial growth on sulfidic materials. Its standard composition is the following (g L^{-1}): $(NH_4)_2SO_4$, 3.70; H_3PO_4, 0.80; $MgSO_4 \cdot 7H_2O$, 0.52; KOH, 0.48. In medium 0Cm, the ammonium concentration was divided by ten. Nutrient media solution (1800 mL) and a representative sample of the coal waste (200 g) were mixed in the reactor until the pulp reached the desired temperature which was then maintained constant until the end of the tests (30 °C for TW30, 42 °C for BRGM-KCC, 48 °C for TW48). The inoculation was performed at 10% (v/v) by adding 200 mL of the above-described cultures in the first reactor R2L1. Then, the pulp of one reactor was used to inoculate the next one. The agitation speed of the impellers was set to 700 rpm in order to maintain the pulp in suspension. The gas flowrate was set to 30 L/h. It is assumed that this flowrate is far above O_2 consumed by the system even in growth phase, which was confirmed regularly by measurement of dissolved O_2. At the end of each batch test, the leached pulp was collected and filtered with a Büchner funnel to separate liquid and solid phases. The filtered solid material was then rinsed with a sulfuric acid solution at pH 1.8 and dried whereas the liquid fraction was stored at 4 °C prior to analysis. The chemical characteristics of both fractions were used to perform a complete mass balance of the leaching operation and to assess the final metal yields.

2.3.3. Reactor monitoring and chemical analyses

Temperature, pH, redox potential, were measured daily directly in the pulp. pH of the pulp was adjusted daily by adding sulfuric acid until the pH stayed below 1.8. Water lost through evaporation was replaced manually every day. Pulp samples were collected daily in the bioreactor, filtered through a 0.45 μm pore-sized filter and stored at 4 °C. Total iron concentration was measured by atomic absorption spectroscopy (Varian SpectrAA-300) in the supernatant fraction. Ferrous iron concentration was estimated using the method proposed by [28]. The chemical composition of the solid residue obtained at the end of each bioleaching test was determined using X-Ray Fluorescence (XRF; Zétium from Panalytical) for Al_2O_3, CaO, Fe_2O_3, K_2O, MgO, MnO, Na_2O, P_2O_5, and SiO_2, by potentiometry for Cl and NH_4, by gravimetry for S^0 and SO_4, by combustion for total S. Sulfide concentration was deduced by subtracting S° and SO_4 concentrations from total sulfur. Chemical composition of the final filtrate was determined after a filtration at 0.45 μm, by ICP-AES for Fe and ionic chromatography for SO_4 and Cl. ICP-AES analysis were realized according to the French standard NF EN ISO11885 on a Jobin Yvon Ultima 2 apparatus. Ionic chromatography was performed following NF EN ISO 10304–1.

2.3.4. Bacterial community monitoring

For the extraction of microbial genomic DNA, two mL of homogeneous pulp from reactor tests were centrifuged (10 min, 14,500 g) within 1 h after sampling. Pellets were washed by re-suspension in 1 mL of 100 mM Tris buffer (pH 8), 2 to 3 times until the pH reached 7 (measured with pH papers), for neutralizing acidity. Pellets were kept frozen at −20 °C until DNA extraction. Microbial genomic DNAs were extracted from frozen pellets with the FastDNA Spin Kit for Soil (MP Biomedicals), according to the manufacturer's instructions, using a speed of 5.0 $m.s^{-1}$ during 30 s and centrifugation of cell debris during 25 min. Extracted DNAs were quantified using the Quantifluor dsDNA sample kit and the Quantus fluorimeter, according to the manufacturer's instructions (Promega).

CE-SSCP (Capillary Electrophoresis Single Strand Conformational Polymorphism) fingerprints were obtained as previously

described [26] on the V3 region (*E. coli* position 331 to 533) of 16S rRNA genes of members of the *Bacteria* domain, amplified from 1 μL of DNA extracts with the bacterial forward primer w49 (5′-ACGGTCCAGACTCCTACGGG-3′) and the universal reverse primer w34 (5′-TTACCGCGGCTGCTGGCAC-3′) 5′ end-labelled with the fluorescent dye FAM. Peak profiles were realigned thanks to the migration of an internal standard (GeneScan 600-LIZ, Life Technologies), and peak position assignment and comparison with peak profile of available known bioleaching strains were done with the software BioNumerics (AppliedMaths).

For cell counts, free bacteria in the pulp were enumerated using a Thoma counting cell under an optical microscope (x400).

2.4. 16S rRNA gene-targeted metagenomics

Genomic DNA was extracted from samples collected in the waste dump in Janina mine using the Qiagen DNeasy PowerSoil DNA isolation kit, with modification to the manufacturer's protocol in order to maximise extraction yields. Each sample (ca. 750 mg) was extracted into three subreplicates subsequently merged at the end of the procedure. The bead beating step was followed by three freeze-thawing cycles (30 min at −80 °C, 5 min 60 °C). Taxonomic diversity of the microbial community of the coal waste dump was characterised by MiSeq 2x250 bp Illumina sequencing of 16S rRNA gene V4–V5 region using the barcoded, universal primer set (515WF/918WR) [29]. Gene amplification and sequencing has been done by INRAE Transfert (France); briefly, PCR reactions were performed using the AccuStart II PCR ToughMix kit, followed by cleaning (HighPrep PCR beads, Mokascience), and pooled triplicates were submitted for sequencing on Illumina MiSeq instrument at GeT-PlaGe (Auzeville, France). Then, Fastq sequences were processed using the FROGS bioinformatics pipeline [30] implemented into the GenoToul Galaxy platform [31]. In brief, after denoising and primer and adapter removal, paired reads were merged with FLASh (*i.e.* Fast Length Adjustment of Short reads) algorithm [32], and clustered into OTU with SWARM and an aggregation distance of 3. After chimera removal with VSEARCH and filtering for OTU abundance (threshold of 0.00005%), taxonomic affiliation was performed using BLASTn and the 132 Silva database. A heatmap was generated with OTU that represent at least the 2% of reads in at least one sample (23 OTU) and using the Jaccard distance matrix and a Principal Coordinates Analyses (PCoA) as ordination method, implemented in the R software.

3. Results and discussion

3.1. Enrichment of bioleaching consortia

The 33 consortia obtained from the coal production waste were assessed for their ability to generate acid (decreasing pH) and oxidise iron (increasing soluble Fe^{3+} and total Fe concentrations). There were only negligible differences in performance between the 11 samples at each temperature (data not shown). Consortia obtained by enrichment at 42 °C were characterized by low rates of acid generation and iron oxidation, as well as low cell abundances. In contrast, the enrichments at 30 °C and 48 °C showed broadly similar rates of iron oxidation and dissolution and final pH. There was a longer lag phase for the 48 °C enrichments, which may be expected given that the ambient temperature of the waste dump surface was relatively cool, having been sampled in winter (mean winter temperature less than 10 °C) and the samples were stored at room temperature prior to the preparation of the enrichments. As the leaching kinetics of the 11 cultures at each temperature were similar, they were pooled to obtain three consortia, namely TW30 at 30 °C, TW48 at 48 °C and TW42 at 42 °C.

Each consortium exhibited a specific bacterial diversity, which was expected since they were enriched and maintained at different temperatures (Table 1). The mesophilic TW30 consortium had the

Table 1
Diversity of *Bacteria* members retrieved in the enriched consortia by 16 S rRNA gene sequencing.

Clone	% in clone library	Closest related microorganism	% of identity by Blastn	Phylum	Main metabolic traits of the related microorganisms	Reference
Members of the TW30 consortium						
TW30-16S13	27.3	*Leptospirillum ferriphilum*	99.8	*Nitrospirae*	Aerobe, acidophile, obligate chemolithoautotroph, oxidise ferrous iron, mesophilic to moderate thermophile with some strains able to grow at 42–45 °C.	[33]
TW30-16S24	12.1	*Leptospirillum ferrooxydans*	99.8	*Nitrospirae*	Aerobe, acidophile, obligate chemolithoautotroph, oxidise ferrous iron, mesophile.	[33]
TW30-16S9	18.2	*Ferrimicrobium acidiphilum*	99.3	*Actinobacteria*	Aerobe, acidophile, obligate heterotroph, oxidise ferrous iron, mesophile.	[36]
TW30-16S11	9.1	*Sulfobacillus thermotolerans*	99.8	*Firmicutes*	Aerobe, acidophile, mixotroph, oxidise ferrous iron, elemental sulfur and sulfide minerals, moderate thermophile.	[37]
TW30-16S4	6.1	*Sulfobacillus* sp.	93.3	*Firmicutes*	Aerobe, acidophile, mixotroph, oxidise ferrous iron, elemental sulfur and sulfide minerals, mesophile to moderate thermophiles.	
TW30-16S17	3.0	*Ferrimicrobium* sp.	91	*Actinobacteria*	Aerobe, acidophile, obligate heterotroph, oxidise ferrous iron, mesophile.	[36]
TW30-16S7	3.0	*Ferrimicrobium* sp.	93.8	*Actinobacteria*	Aerobe, acidophile, obligate heterotroph, oxidise ferrous iron, mesophile.	[36]
TW30-16S14	3.0	*Acidiferrobacter thiooxydans*	99.6	*Proteobacteria*	Facultative anaerobe, acidophile, obligate autotroph, can use ferric iron as terminal electron acceptor, thermotolerant, moderate osmophile rather than halophile.	[38]
TW30-16S2	3.0	*Aciditerrimonas* sp.	90.1	*Actinobacteria*	Facultative anaerobe, acidophile, heteroptroph, able to grow anaerobically or autotrophically by dissimilatory reduction of ferric iron.	[45]

(*continued on next page*)

Table 1 (*continued*)

Clone	% in clone library	Closest related microorganism	% of identity by Blastn	Phylum	Main metabolic traits of the related microorganisms	Reference
Members of the TW48 consortium						
TW48-16S1	51.4	*"Acidithiomicrobium"* P2	99.9	*Actinobacteria*	Aerobe, acidophile, autotroph, oxidise ferrous iron, elemental sulfur and sulfide minerals, moderate thermophile.	[39]
TW48-16S4	17.1	*Sulfobacillus thermosulfidooxidans*	99.6	*Firmicutes*	Aerobe, acidophile, mixotroph, oxidise ferrous iron, elemental sulfur and sulfide minerals, moderate thermophile.	[40]
TW48-16S18	28.6	*Acidithiobacillus caldus*	99.2	*Proteobacteria*	Aerobe, acidophile, mixotroph, oxidise sulfus compounds, moderate thermophile.	[39]
TW48-16S23	2.9	*Sulfobacillus* sp.	93.2	*Firmicutes*	Aerobe, acidophile, mixotroph, oxidise ferrous iron, elemental sulfur and sulfide minerals, mesophile to moderate thermophile.	
Members of the TW42 consortium						
TW42-16S5	73.7	*Sulfobacillus* sp.	99.9	*Firmicutes*	Aerobe, acidophile, mixotroph, oxidise ferrous iron, elemental sulfur and sulfide minerals mesophile to moderate thermophile.	
TW42-16S1	21.1	*Sulfobacillus acidophililus*	99.6	*Firmicutes*	Aerobe, acidophile, mixotroph, oxidise ferrous iron, elemental sulfur and sulfide minerals, moderate thermophile.	[42]
TW42-16S4	5.3	*Acidithiobacillus caldus*	99.2	*Proteobacteria*	Aerobe, acidophile, mixotroph, oxidise sulfus compounds, moderate thermophile.	[39]

largest diversity of OTUs. Among those closely related (sequence identity > 99%) to described bacterial species, the dominant ones (39.4% of OTUs in the clone library) belonged to the *Leptospirillum* genus (*Leptospirillum ferriphilum and L. ferrooxidans*), all being described to date as obligately aerobic chemolithoautotrophs using ferrous iron as sole energy source [33]. *Leptospirillum* strains are generally mesophilic bacteria, although some strains of *L. ferriphilum* are able to grow up to 45 °C [34,35]. The heterotrophic *Ferrimicrobium acidiphilum* (18.2%) is also a mesophilic, obligate iron–oxidizer [36]. Other OTUs were related to iron- and sulfur-oxidising genera: the endospore-forming, mixotroph *Sulfobacillus thermotolerans* (9.1%) and the obligate autotroph *Acidiferrobacter thiooxydans* (3%). Both are described as moderate thermophiles, but are able to grow at 30 °C [37,38].

The diversity was much lower in the consortium TW48, in which three moderate thermophilic species well adapted for growth at 48 °C were found. The autotroph "*Acidithiomicrobium*" strain P2 was largely dominant (51.4%) over the mixotrophic bacteria *Sb. thermosulfidooxidans* (17.1%) and *At. caldus* (28.6%). The two former are iron- and sulfur-oxidizing bacteria while *Acidithiobacillus caldus* is only able to oxidize sulfur compounds [39–41].

The TW42 consortium was mainly (94.7%) composed of *Sulfobacillus* sp. and *Sb. acidophilus* strains, together with a minority of *At. caldus* (5.3%). The instability and very poor growth and iron oxidation of TW42 might be explained by the lack of an efficient autotrophic iron-oxidizing bacterium in this consortium. *At. caldus* oxidizes sulfur compounds but not ferrous iron. *Sulfobacillus* strains are able to grow authotrophically coupling CO_2 fixation to ferrous iron oxidation, but with varying efficiencies. The dominant *Sulfobacillus* strain in the consortium is too distantly related to other species of *Sulfobacillus* to infer its metabolic capacities towards iron. Nevertheless, a gradual decline in the rate of iron oxidation when grown autotrophically and a susceptibility to ferric iron end-product inhibition has been reported for *Sb. acidophilus* strains, together with poor pyrite dissolution for *Sb. acidophilus* strain ALV [42]. Thus, a progressive decline of iron oxidation efficiency during the enrichment procedure cannot be excluded and may have resulted in the poor performances of TW42.

The apparent absence of any strains of *L. ferriphilum* at 42 °C is quite surprising given they are typically considered one of the dominant iron-oxidising organisms in bioleaching operations at these temperatures [35,43], although there is increasing evidence that *Archaea* may play this role in some BIOX operations [44]. Moreover, *L. ferriphilum* was dominant at 30 °C in the TW30 consortium, and so must be present in the waste dump. One hypothesis for its absence in TW42 is that the indigenous strains of *L. ferriphilum* found in the waste dump are less thermotolerant than strains such as *L. ferriphilum* BRGM2 which dominate the BRGM-KCC consortium when grown at 42 °C [43].

3.2. Bioleaching efficiency of TW30, TW48 and BRGM-KCC consortia in bioreactors

The active TW30 and TW48 consortia were used in 2 L reactors and 10% (w/v) solid load to test the predisposition of the pyrite contained in the coal waste to bioleaching at mesophilic (30 °C) and moderate thermophilic (48 °C) temperatures. In addition, as the TW42 consortium exhibited only poor growth and iron oxidation efficiency, the BRGM-KCC consortium, composed of *L. ferriphilum* (dominant), *At. caldus*, *Sb. thermosulfidooxidans* and *Sb. benefaciens* and proven to be efficient for pyrite-rich material bioleaching at 42 °C (*e.g.* [43]), was included in the study to conduct the bioleaching tests at 42 °C. One of the objectives of the CEReS project was to develop and optimise a coal production waste bioleaching process. The majority of commercial tank bioleaching operations run at 40–45 °C, and thus it is important to benchmark bioleaching performance in that range (42 °C in our study) but also at different temperatures (*i.e.* 30 °C and 48 °C).

Pyrite bioleaching mechanisms can be described by the following oxidation reactions:

$$FeS_2 + 2\,Fe^{3+} \rightarrow 3\,Fe^{2+} + 2\,S^0 \quad (1)$$

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

$$S^0 + 3/2\,O_2 + H_2O \rightarrow H_2SO_4 \quad (3)$$

The reactions (2) and (3) are biologically catalysed by acidophilic Fe- and S-oxidising bacteria, whereas the reaction (1) occurs through chemical oxidation.

Monitoring of soluble iron concentrations versus time (Fig. 1) showed typical pyrite bioleaching trend:

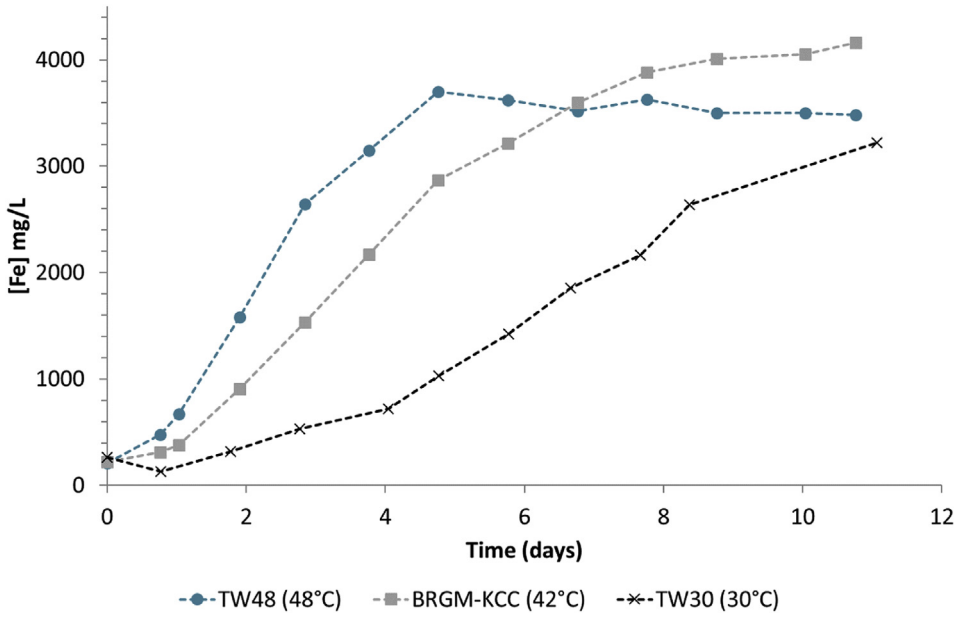

Fig. 1. Soluble total iron over time during the bioleaching of pyritic coal waste in 2 L STR with TW30 (×), TW48 (●) and BRGM-KCC (■). Tests carried out at 10% solid concentration with 0Km medium.

- At first, dissolved Fe concentration remained constant, which corresponds to the microbial lag phase where the biological activity is very low; during this period, the redox was low (below 750 mV), which means that most of Fe is under the form of Fe(II) and pyrite cannot be leached (Eq (1));
- Then, Fe concentration increased sharply, which corresponds to a fast dissolution of pyrite; this period corresponds to the microbial exponential growth phase where the microbial activity is high. During this period, the increase of Fe concentration is mirrored by an increase of the redox potential and a decrease of the pH (data not shown): biological oxidation of Fe(II) and sulfur compounds produced Fe(III) and sulfuric acid according to Eqs (2) and (3), providing the reactants needed for pyrite leaching (Eq (1));
- Finally, Fe concentration stabilized because of the depletion of accessible pyrite, corresponding with a decline in microbial growth and activity.

In this experiment, the two following parameters were considered: 1) the rate of iron release in solution (and thus pyrite dissolution), and 2) the final ferric iron concentration (the yield). These two parameters are essential for process development and scale-up, which rely on a bioleaching culture that is able to dissolve the most pyrite as fast as possible. The Fe dissolution rate increased with increasing temperature (Table 2). The dissolution kinetics obtained with TW30 was very slow compared to the others. Further, after 12 days the soluble ferric iron concentration with TW30 was still considerably lower than the maximum achieved by TW48 after just 5 days. It is unlikely that the poor rate could justify the CAPEX and OPEX of a bioleaching system at this temperature, even if it was able to eventually achieve a similar yield. Therefore TW30 was abandoned. The fastest kinetics was obtained with TW48: Fe concentration reached its maximum concentration (~3700 mg L^{-1}), after only 4.8 days. For BRGM-KCC cultured at 42 °C, the kinetics were slower (3600 mg L^{-1} after 6.8 days) but the final Fe concentration was higher, about 4200 mg L^{-1}. It must be noted that the lag phase was short compared to other studies, which shows that the adaptation methodology derived from [26] was efficient.

Table 2
Summary of bioleaching performances after three subcultures.

	TW48	BRGM-KCC	TW30
Fe dissolution rate (mg Fe/L/h)	45.4	27.8	18.1
Pyrite dissolution yield	80%	71%	50%
Proportion of released Fe which precipitates	16%	0%	0%

The difference between final Fe concentration reached by BRGM-KCC and TW48 might be explained by the precipitation of jarosite [$AFe_3(SO_4)_2(OH)_6$] (A being a cation such as NH_4^+, K^+, H^+) which is a common phase that precipitates in acidic, Fe- and sulfate-rich solutions [46]. Numerous studies have demonstrated that higher temperatures promote higher jarosite precipitation yields [46–48]. To investigate this hypothesis, the iron and sulfide dissolution yields were compared. In the absence of precipitation, both should be equal since pyrite is the only minerals bearing Fe and sulfide. In the presence of precipitation, Fe leaching yield is lower and the proportion of dissolved Fe that precipitates under the form of jarosite can be calculated from the difference between both leaching yields. The results are gathered in Table 2 and show that no precipitation occurred with TW30 and BRGM-KCC whereas, with TW48, 16% of released Fe precipitated.

The 0Km medium was derived from the 0K medium of [49], for the bioleaching of an arsenopyrite concentrate and subsequently used during the development of the Kasese cobalt bioleaching operation [27,50]. The Kasese mineral contains 70% pyrite, and so a higher ammonium content is needed to support the greater microbial activity and biomass production. The Janina spiral tails (about 12% pyrite) are a poorer energy source and so less biomass will be produced during bioleaching and so less ammonium is required. Therefore, further tests were performed with TW48 and BRGM-KCC with a nutrient medium containing less ammonium (ammonium concentration is 0.006 mol L^{-1} in 0Cm medium and 0.056 mol L^{-1} in 0Km medium) to assess the influence of ammonium concentration on precipitation and bioleaching efficiency. The ammonium concentration in 0Km medium was decreased down to the value used in the MSM medium for the enrichment cultures. As

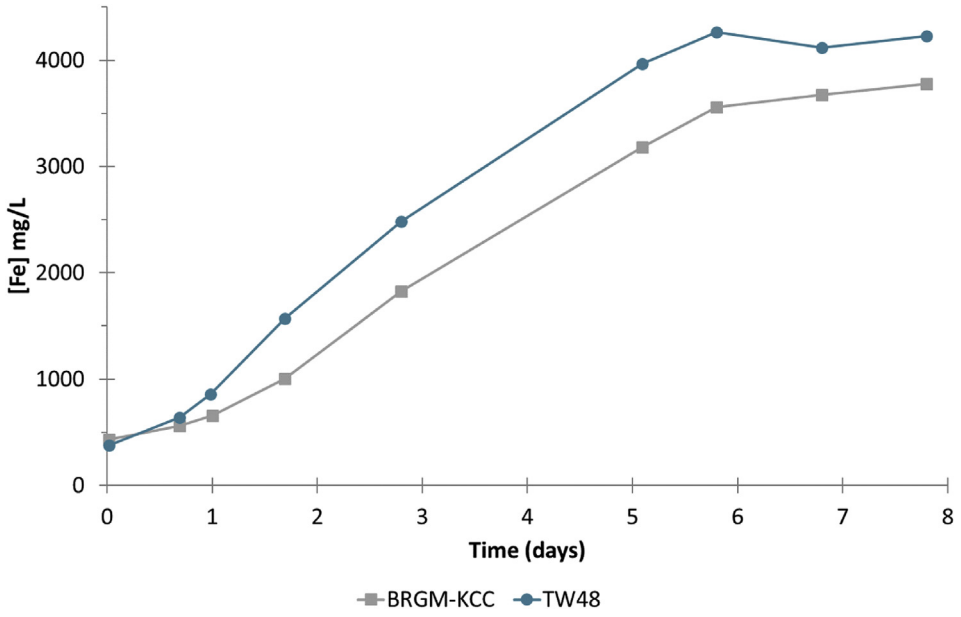

Fig. 2. Soluble total iron over time during the bioleaching of pyritic coal waste in 2 L STR with TW48 (●) and BRGM-KCC (■). Tests carried out at 10% solid concentration with 0Cm medium (ammonium concentration divided by 10).

can be seen in Fig. 2, the Fe dissolution kinetics were similar to the kinetics obtained in 0Km medium, but the final Fe concentration reached with TW48 was higher in 0Cm medium than in 0Km medium (respectively 4230 mg L^{-1} and 3480 mg L^{-1}). The final sulfide and iron leaching yields reached with 0Cm medium were similar, suggesting that Fe precipitation was negligible. Redox potential and pH exhibited a similar trend with both media, which shows that the microbial activity was not affected by the decrease of ammonium concentration.

3.3. Bacterial community evolution in the batch reactor experiments

Routine monitoring of microbial community structure and dynamics of the 2 L bioreactors was done using 16S rRNA gene fingerprinting (CE-SSCP) for the two most efficient consortia, *i.e.* TW48 at 48 °C and BRGM-KCC at 42 °C. There were two aims: to follow the evolution of bacterial community structure during the progressive adaptation at 5% then 10% coal waste concentration (the reactors were run in series, with each reactor being used to inoculate the next), and to identify and compare the bacteria responsible for pyrite bioleaching in the two moderate thermophilic conditions.

For TW48, diversity fingerprints showed a clear dominance of "*Acidithiomicrobium*" P2 over *Sb. thermosulfidooxidans* in the inoculum, as already reported from retrieved 16S rRNA sequences (Table 1), as well as in the two first reactors in the series operated at 5% et 10% solid concentration, respectively (Fig. 3). An evolution of the diversity fingerprints toward a more even distribution of the two strains was observed in the next two reactors in the series: the third reactor also operated in the same conditions, and in the fourth reactor in which the 0Cm medium with 10-fold less ammonium was used.

It is noticeable that the BRGM-KCC reactors at 42 °C, although initially inoculated with *L. ferriphilum* in majority, also showed an evolution towards a community dominated by "*Acidithiomicrobium*" P2 and *Sb. thermosulfidooxidans* (Fig. 3). At 5% and 10% solids with 0Km nutritive medium, the relative abundance of *L. ferriphilum* was too low to be detected on fingerprint profiles, indicating only very low numbers, and a progressive loss of the this organism from the community. Although *L. ferriphilum* was again detected in the next reactor at 10% solids and 0Cm nutritive medium, it was not the dominant strain. Moreover, it was not detectable in subsequent reactors in the series where *Sb. thermosulfidooxidans* B1 became the main bacterium (data not shown), thus confirming the poor development of *L. ferriphilum* during bioleaching of the coal waste. These results are interesting since *L. ferriphilum* is usually the main iron oxidizer developing at 42 °C in bioleaching experiments with BRGM-KCC [43]. Unlike with the enrichment cultures, the strain of *L. ferriphilum* in the BRGM-KCC consortium grows well at 42 °C. Therefore, its loss from the reactors with time cannot be explained by poor thermotolerance.

Such failure to maintain stable development of *L. ferriphilum* on the coal waste material may indicate some form of accrued inhibition. *L. ferriphilum* is a very efficient iron oxidizer, which is expected to improve bioleaching if able to develop and maintain its activity in reactors. Chlorine is present in the coal wastes at concentrations up to 3% (w/w), due to the use of saline water during mineral processing and the coal seam itself is located within a saline aquifer [51]. However, inhibition due to salt dissolution from coal is unlikely, since the highest concentration of chloride observed in the reactors at 10% (w/v) solids was found to be less than 13 mM, well below the range normally tolerated by this organism.

At the same time, significant corrosion of the stainless steel components of the bioreactors was observed, resulting in a linear increase in dissolved nickel concentration, up to 70 mg L^{-1} after 10 days. This would also have led to the dissolution of roughly quantities of chromium (based on typical compositions of 316 L stainless steel). It was hypothesized that the dissolution of chloride ions from the coal waste caused autocatalytic galvanic processes leading to corrosion in localized areas. This phenomenon is known to occur in alloys protected by passivating oxide film, such as stainless steels, in the presence of Cl^- and to lead to the creation of extremely small holes at the exposed surfaces (*i.e.* "pitting corrosion") or gaps at the contact areas between parts (*i.e.* "crevice corrosion") [52].

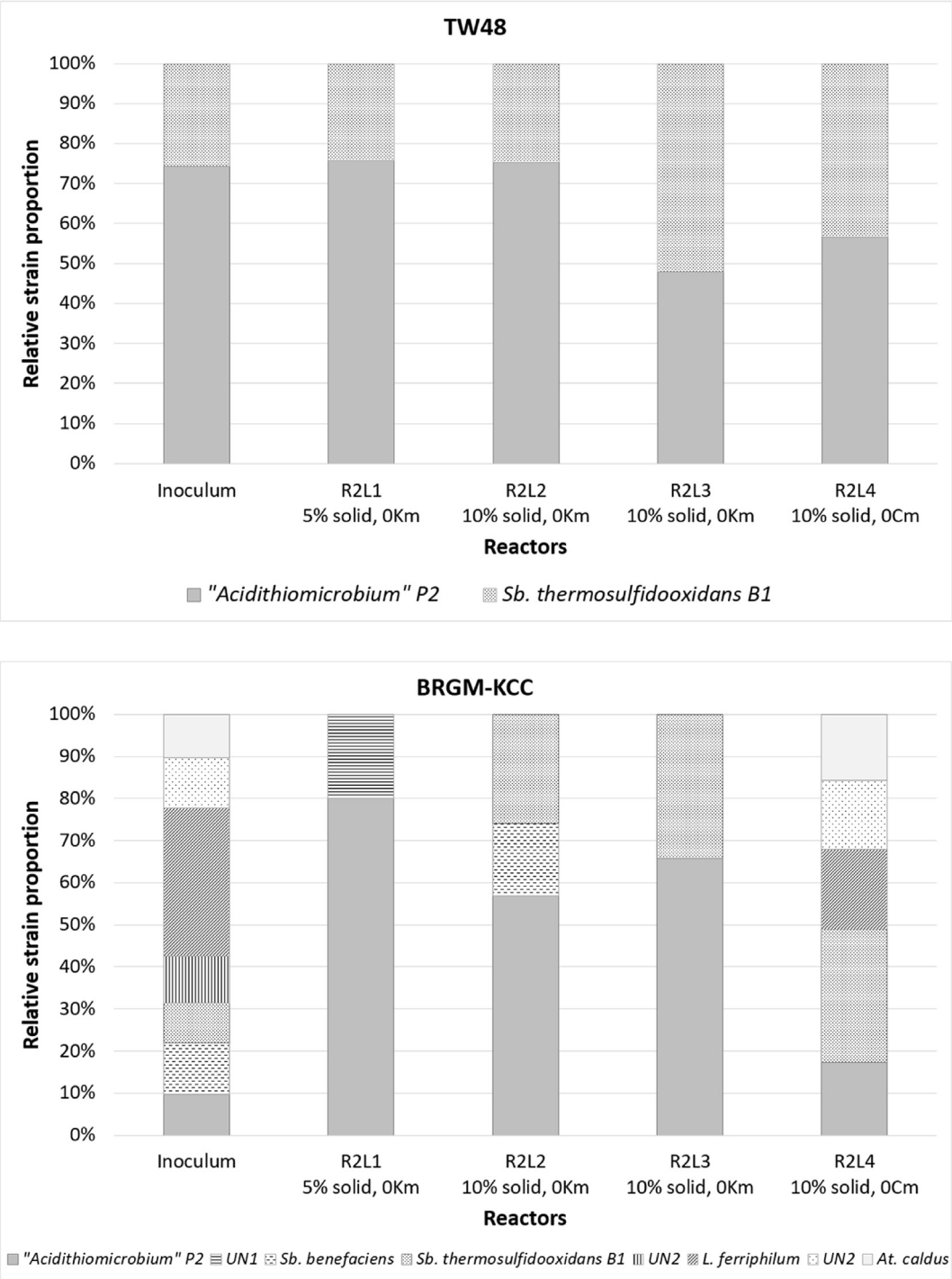

Fig. 3. Diversity figerprints in the TW48 and BRGM-KCC 2 L reactors operated in series at 5% or 10% solid concentrations, and medium 0Km or 0Cm.

The linear increase of Ni in solution measured in the reactors is typical of leaching mechanism led by surface limitation phenomenon which can occurs in our experimentations [53,54].

Previous work has shown that the relative abundance of *L. ferriphilum* in the BRGM-KCC consortium is significantly reduced in the presence of Ni, suggesting it may be particularly sensitive to this metal [55]. At the same time, this was at Ni concentrations of 2.3 g L^{-1}; nearly 50 times higher than those observed in the bioleaching reactors. Analysis of a long-term continuous bioleaching system processing a nickel-copper concentrate indicated that the system was dominated by *Sb. thermosulfidooxidans* and another *Sulfobacillus* sp. with very low levels of *L. ferriphilum* despite having been inoculated with the BRGM-KCC culture (Bryan, unpublished data). Again, this was at Ni concentrations far higher than those seen in the 2 L reactors, but may support a hypothesis that *L. ferriphilum* is un-competitive in nickel-containing systems. Work by Nurmi et al. [56] has shown that while the specific iron oxidation rate of a *L. ferriphilum*-dominated culture decreased with increasing soluble Ni concentration, the concentrations encountered in this study should not cause significant inhibition. There are far fewer

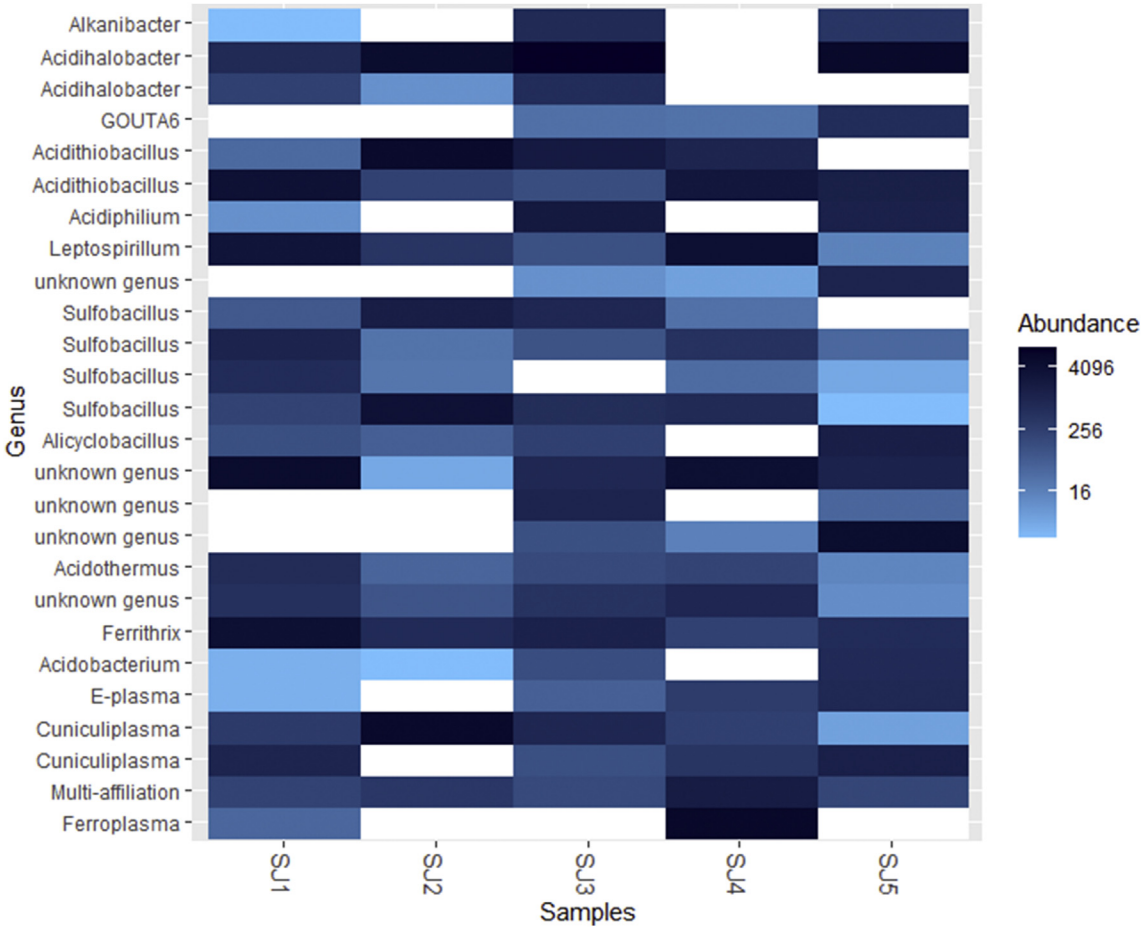

Fig. 4. Heatmap of β-diversity (Jaccard distance) of samples collected in Janina mine waste dump. Selected OTUs were present at least at 2% of reads in at least one sample. Ordination method: PCoA. NA values are in white.

data available on the inhibition of *Leptospirillum* spp. by soluble chromium. However, Johnson et al. [57] have shown that concentrations of Cr^{3+} over 0.52 mg L^{-1} will inhibit the growth and activity of *L. ferriphilum*. No analysis of soluble chromium concentrations was done in this study (although analysis of other reactors affected by the same corrosion issue indicate roughly similar soluble Ni and Cr concentrations; Fonti, unpublished data), and there are significant differences in toxicity of different chromium species.

Leptospirillum spp. dominated the 30 °C enrichment cultures, and so they are clearly capable of colonising the material in bioleaching systems, at least initially; the TW30 cultures were not run in series for as long as the other two consortia and so its long-term survival after multiple sub-culturing cannot be confirmed. At the same time, neither the Cl leached from the mineral, nor the Ni from the stainless steel would appear to have reached concentrations known to be inhibitory. While Cr^{3+} is likely to be far more toxic than Ni, no concentration or speciation data are available. It may be that some other inherent property of the waste is preventing the stable colonisation of the bioleaching reactors by *L. ferriphilum*.

Detailed analysis of the microbial community in the waste dump may give some indications as to whether the material itself inherently favours the development of particular organisms, compared to other types of mine waste or mineral environments. Despite this, efficient oxidation of the pyrite in the coal production waste was achieved in the absence of an obligate autotrophic iron oxidising species by both the BRGM-KCC and TW48 consortia.

3.4. Overview of taxonomic microbial diversity of the waste dump

Retrieved 16S rRNA gene sequences were all assigned to known phyla of the *Bacteria* and *Archaea* domains. Within *Bacteria*, *Proteobacteria* were the most abundant (47.2% of the total sequences), and other phyla representing more than 5% of the diversity were *Actinobacteria* (14.4%), *Firmicutes* (14.0%), *Chloroflexi* (7.9%), *Bacteroidetes* (5.5%) and *Nitrosospira* (5.3%). Rarefaction curves at the genus level reached a plateau for all samples (data not shown), confirming that the number of sequences was large enough to cover the diversity of phylotypes present in each sample. Despite a certain variability among sampling locations, samples shared several OTU (Fig. 4). Within *Bacteria*, most represented OTU belonged to genera typically found in metal rich, low pH environments [5]: iron- and sulfur-oxidizers *Sulfobacillus* (mixotroph, extreme acidophile, *Firmicutes*), *Acidithiobacillus* (mixotroph, extreme acidophile, *Betaproteobacteria*), dissimilatory iron-reducer *Acidiphilum* (heterotroph, moderate acidophile, *Alphaproteobacteria*), iron-oxidizers *Leptospirillum* (autotroph, extreme acidophile, *Nitrospirae*), *Alicyclobacillus* (mixotroph, acidophile, *Firmicutes*), and *Ferrithrix* (heteroptroph, acidophile, *Actinobacteria*). In line with chlorine content in the samples (0.9–7.6 ppk), because of a coal seam located in a saline aquifer and the use of saline water during coal mine process [51], a halotolerant genera commonly found in saline environements, *Acidihalobacter*, was found in large proportion in the dump. *Acidihalobacter* spp. are well adapted to mine environments as species are mesophilic and

chemolithotrophic halotolerant acidophiles able to oxidise iron, sulfur compounds and sulfide minerals at low pH under saline conditions [58]. Within *Archaea*, the genera *Cuniculiplasma* and *Ferroplasma* were found on site. *Cuniculiplasma* is described as an extreme acidophilic, mesophilic, obligate heterotrophic archaeon, unable to oxidize iron despite its widespread occurrence in iron-rich environments such as AMD and sulfidic ore deposits where iron biooxidation is a predominant function [59]. *Ferroplasma* spp. have been isolated from mining sites and are described as major actors in sulfur and sulfide metals cycling in highly acidic environments [60].

The microbial community colonising the waste dump is thus adapted to the main characteristics of the coal mine waste environment and shows a high implication in the oxidation of iron and sulfur. Recently, Sun et al. [61] studied the microbial diversity of a coal mining waste dump and an adjacent AMD creek. They found diverse iron-metabolising bacteria and conclude that coal mining dump may be an important habitat for biogeochemical iron cycling. Their analyses also revealed that pH was the most important environmental parameter influencing microbial community and diversity, as well as links between taxa such as *Acidimicrobiales* and iron- and sulfur-oxidising bacteria such as *Sulfobacillus* spp. It opens perspectives to enhance our study, by exploring in more detail the spatial microbial diversity of the studied heap in link with geochemical parameters, and obtain additional data on endogenous biodiversity and its role in such environments.

4. Conclusion

Coal wastes bearing pyrite are responsible of the formation of AMD detrimental to the environment. Bioleaching has been studied as a way to remove the AMD generating potential of a pyritic coal waste, as initial step of a generic co-processing strategy to reuse and recycle coal wastes.

Cultivation of acidophilic microorganisms from the coal waste resulted in two well-established endogenous consortia each thriving at a specific temperature; 30 °C or 48 °C. Various genera able to oxidise iron and/or sulfur were enriched; *Leptospirillum*, *Ferrimicrobium*, *Sulfobacillus* and *Acidiferrobacter* at 30 °C, and "*Acidithiomicrobium*", *Sulfobacillus* and *Acidithiobacillus* at 48 °C. Both consortia were tested for their capacity to bioleach the pyritic coal waste at their specific temperature in 2-L stirred bioreactors; the highest yield, 80% of pyrite dissolution, was obtained at 48 °C compared to only 50% at 30 °C. In complement, the exogenous BRGM-KCC consortium at 42 °C showed also good bioleaching yield (71%). At 48 °C, iron precipitation under the form of jarosite was observed, but could be easily avoided using a nutritive medium with 10 times less ammonium without any detrimental effect on pyrite dissolution. This result emphasises the importance of looking at nutrients needs and their optimisation for efficient bioleaching. At both temperatures, diversity fingerprints showed an evolution towards a community dominated by the moderate thermophilic bacteria "*Acidithiomicrobium*" P2 and *Sb. thermosulfidooxidans*. If the latter has already been observed at 42 °C, we show here that "*Acidithiomicrobium*" P2 is also able to develop at a temperature lower than usually reported (around 50 °C).

Cultivation of efficient bioleaching consortia from the coal waste failed at 42 °C, a temperature at which the growth of moderate thermophilic strains of *L. ferriphilum* was expected. If the presence of *L. ferriphilum* on site is confirmed by the development of a strain of *L. ferriphilum* at 30 °C, moderate thermophilic strains of this genus may not be present or cultivable from the site. Furthermore, *L. ferriphilum* did not colonise in the BRGM-KCC 2 L bioreactors at 42 °C, although it was the main strain inoculated in the reactor and is usually dominant during pyrite bioleaching with this consortium. Ni (probably Cr) accumulated in the reactors, due to stainless steel components corrosion, and the poor development of *L. ferriphilum* may support a hypothesis that *L. ferriphilum* is un-competitive in nickel-containing systems. On the other hand, these issues may be caused by some other, as yet unidentified, aspects of the waste material, further work needs to be done to determine the precise causes.

Altogether, results confirmed that the studied pyritic coal waste harbour various acidophilic microorganisms known for their bioleaching potential, and the predisposition of the pyrite to bioleaching mesophilic (30 °C) and moderate thermophilic (42 °C and 48 °C) temperatures with endogenous and exogenous consortia. The good leaching yields obtained at moderate thermophilic temperatures open the perspective to optimize further and scale-up the bioleaching process.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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