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EXPLORING THE MICROBIAL COMMUNITY INHABITING THE PHOSPHOGYPSUM STACKS OF HUELVA (SW, SPAIN) BY A HIGH THROUGHPUT 16S/18S rDNA SEQUENCING APPROACH.

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ABSTRACT

Around 100 Mt of phosphogypsum (PG) have been deposited in large stacks on the salt marshes of the Tinto River estuary in Huelva (SW Spain), covering about 1000 ha. These stacks contain extremely acidic water (pH < 2) with high concentrations of pollutants which can cause emissions into their surroundings, generating important environmental concerns. Despite many chemical, geological or hydrological studies have been conducted to characterize the PG stacks of Huelva, the microbial community inhabiting this extreme environment remains unexplored. Using a 16S/18S-rRNA- high throughput sequencing approach, we have uncovered the main taxonomic groups able to live in the acidic metal-contaminated water, which is in direct contact with the PG. In addition, the physicochemical characteristics of the water sampled have been analyzed. These studies have revealed that the most abundant bacteria found in two different leachate samples of the PG stacks belong to the genera *Acidiphilium, Pseudomonas, Leptoprilium, Acidithrix or Acidithibacillus,* which in total represent around 50% of the total bacterial community. These iron-oxidizing and/or reducing bacteria are typical acidophilic genera usually found in acid mine drainage (AMD) environments. Biodiversity of eukaryotes in PG water is lower than that of prokaryotes, especially in the water collected from the perimeter channel that surrounds the PG stacks, where the pH reaches a value of 1.5 and the activity concentrations exceed 300 Bq L^{-1} for ^{238}U or 20 Bq L^{-1} for ^{210}Po, values which are from four to five orders of magnitude higher than those usually found in unperturbed surface waters. Even so, an unexpected diversity of algae, fungi and ciliates have been found in the PG stacks of Huelva, where chlorophyte microalgae and basidiomycetes fungi are the most abundant eukaryotes. Additional bioinformatics tools have been used to perform a functional analysis and predict the most common metabolic pathways in the PG microbiota.

Highlights

- The extreme conditions of these PG stacks hide an unexpected microbial diversity
- The prokaryotic profile includes acidophilic bacteria found in AMD environments
- Archaea, cyanobacteria and sulfate-reducing bacteria are practically absent
- High presence of microalgae in the piezometer denotes seawater influence
- The eukaryotic population in the PG is different from that of the AMD environments

Keywords: 16S/18S rRNA; Acid mine drainage; High throughput sequencing; Metabarcoding; Pollutants; Phosphogypsum; Radionuclides.

1. INTRODUCTION

The phosphoric acid is mainly obtained from the phosphate rock (PR) by treatment with dilute sulfuric acid in the so-called “wet process”. In this process, a by-product called phosphogypsum (PG), which basically consists of gypsum ($CaSO_4 \cdot 2H_2O$) but also contains significant concentration of harmful pollutants, is generated. About 300 Mt yr^{-1} of PG are worldwide produced, being most of it dumped in big waste stockpiles usually placed in coastal zones.

Near the city of Huelva, in the southwest of Spain, around 100 Mt of PG have been generated from 1968 to 2010 by the fertilizer manufacturing companies operating in the area for more than forty years. This huge amount of PG was transported as an aqueous slurry and stockpiled, without any prior treatment or insulation layer, over the salt marsh sediments located in the common estuary formed by the confluence of the Tinto and Odiel Rivers, covering more than 1000 ha and generating a significant environmental impact (Guerrero et al. 2021a). Until 1998, about 20% of the PG generated was directly discharged into the Odiel River channel, while the remaining product was mixed with seawater and pumped into the stacks, where PG was decanted, and the polluted acidic seawater was released into the Tinto River channel without any treatment. After 1998, all the PG generated until the end of the phosphoric acid production activity, in December 2010, was pumped with freshwater in a closed circuit and placed into the piles to comply with the regulations of the OSPAR Convention (OSPAR, 2002).

The PG stacks of Huelva are currently divided in four zones (Fig. 1). Zones 1 and 4, with a total extension of around $5.5 \cdot 10^6$ m^2 and located at the north and south of the affected area, respectively, are already considered as restored. However, polluted edge outflows from these two zones have been detected reaching the water of the Tinto estuary (Pérez-López et al., 2016). In Zones 2 and 3, the uncovered PG is directly exposed to weathering and contains superficial acidic water ponds, shown in green color in Fig. 1. Depth and extension of these ponds are progressively decreasing by evaporation during the

ongoing restoration of the area. In zone 2, about 25 Mt of PG are accumulated covering a surface of around $2.5 \cdot 10^6$ m^2 and forming a pyramidal pile of up to 30 m high, surrounded by a network of perimeter channels to collect the leachates from the stack. The PG stacks of Huelva have been exhaustively studied from the geochemical point of view with particular attention to the pollution pathways. Aspects such as the mobility of heavy metals and radionuclides contaminants present in the PG (Pérez-López et al., 2018; Guerrero et al., 2020) or the composition of the leachates released from the stacks (Pérez-López et al., 2016; Papaslioti et al., 2018; Guerrero et al., 2021a) have received special attention. However, to date, the microbial community that inhabits the PG piles of Huelva has not been explored at all. The extremely toxic composition of the PG, besides the adverse conditions for life (high solar irradiance and salinity, low water content, and extremely low pH) have probably discouraged research in this sense.

PG contains impurities such as fluoride, diluted phosphoric acid, heavy metals, or radionuclides coming from the original phosphate rocks as natural contaminants (Bolívar et al., 2009; Guerrero et al., 2020), which increase the potential environmental impact of PG and limit the proliferation of microorganisms. U-series radionuclides, heavy metals, such as Cd or Ni, and metalloids as As are naturally present in most phosphate ores, and they are transferred into the PG during the production of the phosphoric acid (Bolivar et al., 2009). The high mobility of these pollutants at the low pH values of the PG leachates causes their easy dissolution. Therefore, the outflows coming from the PG piles or their pore waters contain very high concentrations of heavy metals and radionuclides, which can reach concentrations from 3 to 5 orders of magnitude higher than those observed in unperturbed waters, as we have recently demonstrated (Guerrero et al., 2021a)..The presence of such pollutants, the acidic nature of PG and the high proportion of compounds with biocide activity make the physiological conditions of PG highly inhospitable. However, there are many examples of extreme environments where microorganisms are able to proliferate, becoming adapted to acidic or basic pH values,

high concentrations of NaCl, heavy metals, organic solvents, or other xenobiotics, and demonstrating the adaptation ability of microbial life to colonize a wide range of extreme ecological niches, and the PG stacks of Huelva are not an exception (Merino et al., 2019).

There are PG deposits at several locations worldwide, especially near the main phosphoric acid production plants, in China, USA, Tunisia, or Morocco. However, the microbial species able to thrive under these adverse conditions and flourish in contact with the PG have remained practically unexplored. The few studies published in relation to the microbiology of PG have been carried out in the deposits of Gafsa and Sfax, in Tunisia (Mefteh et al., 2019; Trifi et al., 2020). These studies were focused on the microbiota associated with the PG sediments and with certain plant species able to grow in the vicinity of the PG. However, the microbial community present in the leachates of these PG stacks or in the PG deposits of other locations has not been investigated to date.

2. MATERIALS AND METHODS

2.1. Sampling

The two PG leachate samples used in this study were collected at the beginning of July, before the total loss of water by evaporation that usually takes place at the end of the annual hydrological cycle. The first water sample was taken from the perimeter drainage channel (Fig. 1, code “PC”) that surrounds the Zone 2 of the stacks. The second one was taken from a piezometer (Fig. 1, code “PZ”) that collects the groundwater from the south border of the same zone, around 3-4 m in depth. The UTM coordinates (WGS84 datum) for the sampling points in the channel and the piezometer are 29 S 684521, 4123390, and 29 S 684536, 4123295, respectively. Seawater, sampled near the city of Huelva, and water of the Tinto River, collected at the lower course of the river (near the town of Niebla) have also been included for comparison. The water samples were collected by an electrical pump with a flow rate of 4000 L h^{-1} in sterile 25 L plastic tanks and transported to the lab for their immediate processing.

2.2. Determination of physicochemical parameters of the leachates

Physicochemical parameters as electrical conductivity (EC), pH, oxidation-reduction potential (ORP), and temperature (T), were determined *in situ* by a Hanna HI98195 portable multimeter, with a HI7698195 multiparameter pH/EC/T probe. The pH/ORP sensor used (ref. HI7698194-1) contains a glass pH sensing tip combined with an Ag/AgCl reference electrode in gel electrolyte. The instruments were calibrated before sampling, and the ORP was corrected to obtain the potential relative to the hydrogen electrode (Eh).

2.3. Chemical and radiochemical analysis of the leachates

To determine the metallic composition of the water, the samples were digested with HCL at 150ºC during 30 min and analyzed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) using an Agilent 5110 spectrometer, equipped with a SeaSpray nebulizer. The argon line was used as an internal standard f. The quality control (QC) was implemented by the measurement of Certified Reference Materials (CRMs). To determine the anionic composition, the water samples were filtered through a 0.45 µm filter and analyzed by ionic chromatography in a Methrom 882 Compact IC plus instrument, equipped with an 858Pro auto-sampler, a cationic suppressor, and a Metrosep A-Supp 5-150/4-0 column. The mobile phase consisted of sodium carbonate (0.339 g L^{-1}) and sodium bicarbonate (0.252 g L^{-1}). Flow rate: 1 mL min^{-1}. Injected volume: 20 μL^{-1}. All measurements were done in triplicate.

Natural radionuclide concentrations in the collected leachates were determined by a sequential extraction technique based on the use of tributyl phosphate (TBP), subsequent electrodeposition onto stainless-steel disc (U-Th-Ra isotopes), and self-deposition onto silver discs for the case of ^{210}Po. The radioactive sources were counted by α-particle spectrometry using ion-implanted silicon detectors, with a 25% absolute efficiency. The QC for alpha-particle measurements was conducted by participating in annual international proficiency tests (International Atomic Energy Agency [IAEA] and the Spanish Nuclear Safety Council [CSN]), and by measuring both a blank and CRMs (IAEA-434) every set of six samples (Bolívar et al., 2009).

2.4. DNA isolation

For genomic DNA isolation, 10 L of PG water samples, were filtered through a 0.7 µm glass fiber filter (Whatman, GF/G), and the retained biomass was later eluted by backflushing with some additional sample water. Finally, the concentrated samples were centrifuged at 12,000 xg and the biomass obtained was used for genomic DNA extraction

with the GeneJET Genomic Purification kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions.

2.5. Library preparation and DNA sequencing

Prokaryotic and eukaryotic rDNA amplicons were obtained by PCR with the Phusion® High-Fidelity PCR Master Mix (New England Biolabs, MA, USA), using the genomic DNA previously isolated as a template and the primer sets: 341F/806R, to amplify the hypervariable V3-V4 16S rDNA region; and 1380F/1510R, to amplify the V9 18S rDNA region (supplementary material, Table S1). The PCR products were cleaned up using the Qiagen Gel Extraction Kit (Qiagen, Germany) and pooled to generate two libraries, one prokaryotic and one eukaryotic, using NEBNext® UltraTM DNA Library Prep Kit for Illumina and quantified accurately with the Qubit and Q-PCR. Library pools were sequenced in the Illumine MiSeq platform using the Illumina MiSeq Reagent kit V2x 250bp, to generate paired-end raw reads.

2.6. Bioinformatic data processing and OTUs clustering

Bioinformatic analysis of the data was carried out using QIIME2 v2020.8 (Bolyen et al., 2019). Briefly raw data were demultiplexed, using the q2-demux plugin, and filtered by trimming and truncating low-quality regions, eliminating replicated reads, and filtering chimeras with DADA2 (via q2-dada2) (Callahan.et., 2016). The reads were then clustered in operational taxonomic units (OTUs) using a 97% similarity cutoff, with the *de novo* clustering method (via q2-vsearch) from VSEARCH (Rognes et al., 2016).

2.7. Taxonomic classification and Functional analysis

OTUs were classified at each taxonomic rank using the *q2-feature-classifier* plugin (via *classify-sklearn* method) and the SILVA database (Quast et al., 2013) with two different pre-trained classifiers, specially curated for 16SV3V4 and 18SV9 regions. Annotation was performed with a 0.7 threshold. The taxonomic information retrieved for the best hits was used for the annotation at different taxonomic levels (kingdom, phylum, class, order, family, genus, and species, if possible). For the analysis of the species diversity, the *alpha-rarefaction* tool was used via q2-diversity to generate a rarefaction curve as previously described (Gómez-Villegas et al., 2018).

Finally, the inferential functional analysis was performed using PICRUSt2 (Douglas et al., 2020). The entire PICRUSt2 pipeline was used to generate metagenome predictions from 16S rRNA amplicon through sequence placement, and pathway-level prediction by predicting EC numbers, KEGG orthology terms (K numbers), and MetaCyc pathways. KEGG pathway maps were obtained from the generated set of K numbers using the KEGG Reconstruct tool (update: July 1, 2021). Venn Diagrams were generated using the VennDiagram package (v.1.6.20) for RStudio (v1.3.1093).

3. RESULTS AND DISCUSSION

3.1. Hydrochemical characterization of the leachates

The values of the measured physicochemical parameters, the concentration of the most significant elements and anions, as well as the activity concentration of the main natural radionuclides in the two water samples collected from the PG stacks of Huelva, are displayed in Table 1. The values corresponding to the two reference samples (seawater and Tinto River water) have also been included in this table. While the physicochemical parameters and the concentration of dissolved species in seawater remain approximately stable over time, the Tinto River shows a strong variability, for this reason, the mean values for a hydrological year were included in Table 1.

The pH measured in the perimeter channel water was extremely acidic, with apH value around 1.6. The Eh indicates the oxidizing conditions of the channel with a value of 674 mV. The EC showed a value of 42 mS cm^{-1} which indicates a high concentration of dissolved ions. The content of contaminants such as As, Cd, or Ni was also very high, with values of 33, 9.8, and 6.3 mg L^{-1}, respectively, which is between 45 fold (for Ni) and 150 fold (for As) the values observed in the water of the Tinto River, which has been included as a reference. The concentrations of other metals, such as Cu, Co, or Fe were of the same order or even lower than in the Tinto River, which is highly influenced by the acid mine drainages. Other hazard contaminants such as Hg or Se were undetectable. The extremely high activity concentration of ^{238}U-series radionuclides at this sampling point is noteworthy. The values exceed 300 Bq L^{-1} for ^{238}U or 20 Bq L^{-1} for ^{210}Po, being from four to five orders of magnitude higher than those observed in unperturbed freshwater and seawater.

The stacks of Huelva stopped receiving PG in December 2010, however maintenance and restoring works are ongoing and the area counts with several piezometers were built

along zones 2 and 3 of the stacks to monitor the phreatic level and to allow the collection of groundwater samples. The location of the sampled piezometer is shown in Fig. 1. It has a total depth of 4.8 m and collects leachates through its last perforated meter. In the point where the piezometer is located, there is 35 cm of restored soil over the PG, which comprises a layer of about 5 m thick. This was our second sampling point, which allowed us to obtain underground polluted water from the bottom of the PG material. The pH of the piezometer water was 1.7, an extremely low value, but slightly higher than that observed in the water of the perimeter channel. The Eh denotes significant oxidizing conditions, but the measured value (480 mV) was significantly lower than the obtained in the channel, due to the fact that this sample has a groundwater source. Besides, the EC value in the piezometer is 53.6 mS cm^{-1}, higher than the value at the channel., The high EC value in the piezometer, very similar to the value of the seawater (Table 1), is due to the influence of the seawater used for transporting the PG until 1997. The concentrations for As, Cd, or Ni in the PZ, with values of 9.2, 2.5, and 1.8 mg L^{-1}, respectively, are much higher than in the Tinto River, but not as high as in the perimeter channel. The concentration of As in the piezometer is almost 50-fold the concentration in the Tinto River, however, this is around three times lower than the value measured in the perimeter channel. Similarly, the concentration of Cd in the PZ is almost 30 times the concentration in the Tinto River and four times lower than in the PC. In general, it can be confirmed that the concentration of all the tested elements was lower in the piezometer than in the perimeter channel, excepting the cations K, Mg, and Na or the anion Cl, which showed higher concentration values in the piezometer due to a clear influence of the seawater. The activity concentration of the analyzed natural radionuclides was also very high at this point, but one order of magnitude lower than in the perimeter channel for the U- and Th-isotopes. The concentration of fluoride, a well-known biocide anion, is particularly high in the perimeter channel water, with a value of 1.4 10^3 mg L^{-1}. The concentration of fluoride in the piezometer water was 85 mg L^{-1} and undetectable in the Tinto River.

All these data indicate that the acidity, the content of toxic heavy metals, natural radionuclides, and fluoride are higher in the perimeter channel than in the undergrown water collected in the piezometer, which, on the other hand, presents higher electrical conductivity and higher salinity due to a clear influence of the seawater. However, in both cases, the low pH values and the high content of toxic metals will condition the microbiota which will have to be specially adapted to thrive with these harsh conditions

3.2. High throughput sequencing of the rRNA markers encoding genes and estimation of biodiversity in the PG water samples

The reads obtained from the high throughput sequencing of the 16S rRNA libraries generated for both PG water samples were denoised, filtered to eliminate chimeras and PCR artifacts, and clustered into 680 and 596 taxonomic units for the perimeter channel and the piezometer water samples, respectively. Sequencing of the 18S rRNA libraries, on the other hand, yield 38 and 186 OTUs, for the perimeter channel and the piezometer water samples, after processing by the same bioinformatics pipeline. The number of sequences and the mean quality score for each sample are shown in Table 2, while rarefaction curves are in Supplementary material (Fig. S1).

It is noteworthy that the volume of water processed to obtain enough genomic DNA was about 5 fold higher in the PG water samples than in the Tinto River or seawater samples. Moreover, the number of raw reads and the number of non-chimeric merged inputs obtained is around one order of magnitude lower in the PG water samples than in the Tinto River or seawater samples. This indicates a lower biomass content in the PG water samples in comparison with the biomass found in the seawater or the Tinto River water, as was expected for the harsh living conditions that prevail in that environment.

The Shannon-Weiner biodiversity index (H) for the whole prokaryotic population, in both the perimeter channel and in the piezometer, are shown in Table 2 and compared with

304 the biodiversity index of the Tinto River or the Sea water. The biodiversity index for the
305 prokaryotic community is higher for the PG water samples, with values of 7.7 and 7.5,
306 than for the control Sea and Tinto River water samples.

307 Similar analysis done for the 18S rRNA libraries revealed that the Shannon biodiversity
308 index in the eukaryotic community is 1.5, in the case of perimeter channel water, and
309 4.5, in the water collected in the piezometer. Although the biodiversity index for the
310 eukaryotic population of the piezometer water is similar to that observed in seawater
311 (4.5), the value obtained for the perimeter channel is lower (1.5), indicating a more
312 reduced biodiversity within the eukaryotic community of the perimeter channel water. In
313 the Tinto River, the Shannon index for eukaryotes (6.3) is higher than that for the
314 prokaryotic population (5.1). In agreement with different previous studies, which have
315 repeatedly confirmed the paradoxical high eukaryotic diversity in the Tinto River, that
316 was even superior to that of prokaryotes (Zettler et al. 2002; Gadanho, et al., 2006;
317 Aguilera 2013).

318 The extreme physicochemical parameters of the PG water suppose an inconvenience
319 for the development of high concentrations of microorganisms, however, do not impede
320 the existence of a large variety of species, especially of the prokaryotic kingdom. The
321 extremely low pH value and the high redox potential of the water from the perimeter
322 channel seem to affect more drastically to the eukaryotic community, which exhibited a
323 very low biodiversity index (1.5) and presented a low number of OTUs.

324

325 **3.3. Prokaryotic population in the water samples collected from the PG stacks**

326 Analyzing the data obtained from sequencing the 16S library, we can conclude that in
327 the PG water, from either the piezometer or the perimeter channel water, the most
328 represented sequences correspond to the phylum Proteobacteria, which supposes 62%
329 of the total OTUs in the perimeter channel and 67% of the piezometer, followed by the

phyla Actinobacteriota and Nitrospirota, which represent around 15% and 5%, respectively, in both PG water samples. These phyla comprise bacteria of very different characteristics, however, it is possible to observe that the phyla profile in the PG water differs significantly from that of the seawater, where Proteobacteria are much more abundant (90%). This profile is also very different from that of the Tinto River, where the phylum Nitrospirota represents 23% of the total (Fig. 2).

Focussing at lower taxonomic levels, the most abundant OTUs in both PG water samples correspond to the classes Gammaproteobacteria and Alphaproteobacteria, which are also the most represented in the seawater and the Tinto River. However, the classes Leptospirillia and Acidimicrobiia which are very abundant in the perimeter channel with percentages of 23% and 7%, respectively, are poorly represented in the underground water of the piezometer. The classes Actinobacteriae and Bacteroidia, by contrast, are more abundant in the piezometer (around 3%) than in the perimeter channel.

If we go down in the taxonomic scale and look at the genus level, *Acidiphilium* is the most abundant one, representing 10.4% of total OTUs in the perimeter channel and about 11.3% in the piezometer. Followed by the genera *Pseudomonas*, *Leptospririllum, Acidithrix, Acidithiobacillus, Stenotrophomonas,* and *Ferrovum*, which suppose between 4 and 5%. of the total OTUs. All these genera count with extremophilic representatives and many of them have been found to be present in AMD environments, where the most abundant taxa include the genera *Acidiphilium, Acidisphaera, Acidithiobacillus*, and *Leptospirillum* (López-Archilla 2001; Lukhele et al. 2020). Most of these genera coincide with the main genera represented in our sample of the Tinto River, in which 25% and 20% of the OTUs correspond to the genera *Acidiphilium* and *Leptospirillum*. The genera *Acidithrix* (6.1%), *Acidithiobacillus* (10.7%), and *Ferrovum* (11.1%) are also present at high percentages in our sample from the Tinto River.

Acidiphilium is an acidophilic genus, usually found in AMD environments This versatile genus has been proposed to have arisen in ancestral non-extreme conditions and have

357 got adapted to harsh acidic environments by the acquisition of new functional features
358 through the evolution. The trait for this adaptive evolution can be observed in the different
359 genomes available for species of the genus, which contain a repertory of genes, acquired
360 by horizontal gene transfer, to cope with metal and osmotic stress or to metabolize
361 different nutrients (Li et al., 2020). In our study, the most represented species of the
362 genus are *Acidiphilium cryptum* (100% sequence homology) and *Acidiphilium rubrum*
363 (99.5% sequence homology), species also found in AMD environments in Canada (Auld
364 et al., 2013) and Turkey (Aytar et al., 2014). From a metabolic point of view, most
365 *Acidiphilium* sp. are aerobic acidophilic heterotrophs that utilize organic compounds to
366 obtain carbon and energy, although many can obtain some extra energy from the
367 mixotrophic oxidation of ferrous iron. In addition, some members of the genus have been
368 described to be able to reduce Fe^{3+}, respiring iron under anaerobic or microaerophilic
369 conditions or to perform photosynthetic CO_2 assimilation (Kisková et al. 2018).

370 On the other hand, the genera *Acidithiobacillus* and *Leptospririllum* comprise a series of
371 obligate or facultative chemolithoautotrophs widespread found in ADM sites. The genus
372 *Acidithiobacillus* is an important representative of the sulfur-oxidizing bacteria. The
373 species of this genus are strict autotrophic Gram-negative bacteria with a versatile
374 metabolism that can fix both carbon and nitrogen from the atmosphere, using ferrous
375 iron or reduced sulfur compounds as a primary source of energy and generating the
376 corresponding oxidized ferric or sulfur species, contributing in this way to the sulfur
377 biogeochemical cycle. They can grow aerobically or anaerobically, using ferric iron as
378 the final electron acceptor. The most abundant species of this genus in our samples were
379 *Acidithiobacillus ferriphilus* and *A. ferrooxidans,* which are important contributors to the
380 sulfur biogeochemical cycle. A low representation of *Acidithiobacillus thiooxidans*,
381 formerly known as *Thiobacillus thiooxidans,* has been found in the PG samples of Huelva
382 in this study. This species is one of the best-known iron-oxidizing acidophiles, very

abundant in the acidic environment of the Rinto River and essential for the recovery of copper by bioleaching (González-Toril et al., 2003).

The genus *Leptospirillum* includes different iron-oxidizing species, which role in the acidic environment has gained an increasing acknowledgment (Méndez-García et al., 2015). In our study, *Leptospirillum ferrooxidans,* which represents 3.4% and 3.7 % of the total OTUs in the perimeter channel and the piezometer, respectively; and *Leptospirillum ferrodiazotrophum*, which suppose around 1% of the total OTUs in both locations, are the most represented species of the genus. These species are present a much higher percentage in the Tinto River water (16.3% for *L. ferroxidans*) and are practically non-existent in the seawater.

Other acidophilic genera found in the Huelva PG samples are *Acidithrix*, and *Ferrovum.* The first species of the genus *Acidithrix* described was *Acidithrix ferrooxidans,* isolated in north Wales from an acid mine drainage site and classified as a new heterotrophic acidophilic species. *Acidithrix*, as other acidophiles, can use Fe^{2+} (but not sulfur) as an inorganic electron donor and an organic carbon source, getting extra energy from this mixotrophic nutrition . Under the limitation of oxygen species of this genus have been described to reduce ferric iron. The reads assigned to Aciditrhix in our study only share 89% of sequence identity with *A. ferroxidans*, however, they have a 100% of sequence identity with other uncultured *Aciditrix* genera identified in metagenomic surveys in locations as distant as the acidic hot springs of the Colombian Andes (Bohorquez et al., 2012), the extreme acidic Pitlakes from the Iberian Pyrite Belt (Santofimia et al., 2013) or polymetallic deposits in Russia (Gavrilov et al., 2019).The species of the genus *Ferrovum* are iron oxidizers chemolithoautotrophs that pair Fe(II) oxidation with the fixation of carbon dioxide as a carbon source although there is a high metabolic diversity within the genus. We have found in the PG a high percentage of OTUs (4 and 4,6%) with high sequence homology with uncultured *Ferrovum* species found in acidic environments.

The genus *Ferrovum* belongs to the order Burkholderia. Some strains of this order, such as *Burkholderia fungorum*- or *Paraburkholderia fungorum strain Rifles*, have been described to respire uranium, anaerobically reducing U (VI) into U (IV) for their growth (Agarwal et al., 2019). Several OTUs assigned to uncultured species of the Burkholderiaceae family have been found in our studies. The sequences corresponding to these OTUs showed an identity of only 90% with the corresponding sequence of *P. fungorum*, however, the high concentration of uranium in the perimeter channel water (Table 1) makes probable the existence in this sample of new linages related or not with *P. fungrum*, with the ability to reduce uranium.

Other abundant genera are *Pseudomonas* and *Stenotrophomonas*, which represented about 4.9 and 4.4%, respectively, of the total 16SrRNA OTUs obtained from the perimeter channel; and about 2% and 5.1% in the case of the piezometer water. The genus *Stenotrophomonas* is widely spread in aqueous environments. Their ability to form biofilms and their versatility can explain the capacity to colonize extreme environments of some species of the genus. Recently, the tolerance of this Fe–Mn oxidizing bacterial genus to high concentrations of iron and its ability to treat AMD effluents has been demonstrated (Hou et al., 2020). However, this is not a typical acidophilic genus, and it is also present at a high concentration in the sea water control sample. *Pseudomonas* is other ubiquitous versatile genus with species adapted to many different extreme conditions, including acidic environments (Kisková et al., 2018).

It is noteworthy that the prokaryotic population present in the PG water comprises almost exclusively bacteria (99.8 %), being the presence of archaea practically null. The acidophilic or sulfur-chemolithoautotrophic archaea described to date are related to hot acid springs and are mainly restricted to the order *Sulfolobales* (Ghosh and Dam, 2009), which is not represented in our study.

Zouch and coworkers found the development of sulfate-reducing bacteria (SRB), such as *Desulfobacter or Desulfovibrio,* in enrichment cultures inoculated with sediments

437 collected from the vicinity of Stockpiled Sfax Phosphogypsum in Tunisia (Zouch et al.,
438 2017), but we found not a trace of these bacteria in the Odiel PG water. Neither,
439 cyanobacteria, which are among the most widespread primary producers both in the
440 oceans and in the freshwater, are present in the PG leachates, although several genera
441 of this group appear in the seawater sample.

442 Comparison of the microbiota found in the Huelva PG leachates samples with that of
443 other similar PG sites is impossible because there are no similar studies. The few studies
444 carried out to identify the microorganisms in the PG areas of Tunisia were not focused
445 on the water but on the sediments or the scarce vegetation that proliferate in the zone.
446 Mefteh and coworkers (Mefteh et al., 2019) concluded analyzing the sediments of the
447 Tunisian PG of Sfax that the dominant phylum was Proteobacteria with more than 40%
448 of the total prokaryotic sequences, being the most abundant genera *Staphylococcus,*
449 *Bacillus, Pseudomonas,* and *Acidithiobacillus*, although important differences were
450 found depending on the bioinformatics pipelines and the databases used for the
451 taxonomic assignment.

452 Trifi and coworkers (Trifi et al., 2020), on the other hand, explored the same PG Tunisian
453 location, focusing on the microbiota present in the PG sediments and associated with
454 four higher plants growing in the contaminated PG vicinity. They found that the most
455 abundant phyla in the sediments were Firmicutes (39.3%), Proteobacteria (24.2%), and
456 Actinobacteria (20.4%) and the dominant genera were *Bacillus* (20.7%) and
457 *Enterococcus* (17.6%).

458 None of these results coincide with the microbial profile that we have found in the PG
459 stacks of Huelva. However, as we have already pointed, the Tunisian study was done in
460 the sediments while we explored the microbial community in the water reservoirs of the
461 PG. In addition, the PG water in Huelva can be highly influenced by the acid mine
462 drainage discharged into the Tinto River, making the microbial community of the Huelva
463 PG stacks a unique microbiota.

3.4. Eukaryotic population in the water samples collected from the PG stacks

The most abundant eukaryotic phylum in the perimeter channel is Basidiomycota, which constitutes 47.3% of the total OTUs obtained (Fig. 3). Specifically, the fungal groups Filobasidiales and Agaricales, were the most abundant orders within this phylum. A more detailed analysis reveals that the fungal species found share a high sequence identity (98%) with the yeast *Solicoccozyma* sp*.* and the widespread fungus *Schizophyllum radiatum*, which are versatile basidiomycetes with genetic and biotechnological interest (Li et al., 2020). However, no acidophilic strains of these species have been described until now. The percentage of fungi (including yeast) within the eukaryotic community of the piezometer leachates is low in comparison with the perimeter channel. The phyla Basidiomycota and Ascomycota only represent 4% and 1.1%, respectively, of the eukaryotic OTUs in the piezometer.

Although several yeast species have been isolated from extreme aquatic environments, such a *Cryptococcus*, *Rhodotorula,* or Lecytophora, found in the Sao Domingos mine in Portugal (Gadanho et al., 2006) and in the Tinto River in Spain (Aguilera 2013), acidophilic strains of the yeast *Soliccoyma* have not been reported in these locations. Another acidic environment in which the eukaryotic heterotrophic community has been deeply studied is the Rio Agrio, a naturally acidic river in the vicinity of a volcanic area in the Argentine Patagonia. In this acidic location, the main acidophilic yeasts described belong, as in the areas influenced by acidic mine drainages, to the species *Cryptococcus* and *Rhodotorula* (Russo et al., 2008). The absence of these typically acidophilic fungal species in the PG leachates is noteworthy. Similarly, different genera of extremophilic filamentous fungi have been found in hyperacidic environments (López-Archilla et al 2004). However, to our knowledge, the existence of acidophilic strains of the fungi *Schizophyllum* in other locations, apart from the PG of Huelva, have not been yet reported. The pH values in the PG piezometer and perimeter channel are around 1 and

1.2 units lower than the medium pH value of the Tinto River water. This suggests that the pH of the PG leachates must be under the threshold pH tolerable by the typically acidophilic fungal species found in other acidic water bodies.

Regarding photosynthetic eukaryotes, the phylum Chlorophyta is the most represented, supposing 18% of the eukaryotes in the perimeter channel and 62.1% in the piezometer (Fig. 3). Chlorophyta is also the most abundant phylum in the seawater and in the Tinto River, where it reaches 40.7% and 81.8% of total OTUs, respectively. The phyla Ocrhophyta (6.6%), Dinoflagellata (1.7%), Diatomea (1.4%), Protalveolata (1.3%), and Cryptophyceae (1%) are also present at significant percentages in the piezometer leachates. Instead, in the perimeter channel the only microalgal phylum of importance, apart from the Chlorophyta, is the Dinoflagellata, which represents 11.1% of the total eukaryotics OTUs. Curiously, the diversity of photosynthetic eukaryotes contrasts the absence of cyanobacteria in the PG leachates and in acidic environments in general.

The phylum Chlorophyta is a highly diverse group of algae closely related to higher plants. Most of the species of this phylum are freshwater, however, there are important marine genera. In the Piezometer water, families of this phylum, such as Treboxinophyceae (48%) and Chlorophyceae (3.2%) are abundant, while in the perimeter channel the most represented chlorophyte group is Chlorodendrophyceae, which includes the genus *Tetraselmis*. In fact, OTUs which share 90-93% sequence identity with several species included in the database as *Tetraselmis* sp. or unclassified Prasinophycea are highly represented in the perimeter channel.

Comparison with the eukaryotes present in other PG deposits is not possible, because none of the studies done in other PG affected areas, such as those realized in Tunisian PG, have investigated the eukaryotic population (Mefteh et al. 2019; Trifi et al. 2020). Once again, the most related environments are those affected by acid mine drainages or volcanic activity. The eukaryotic community of the Tinto River is one the best studied in an extremely acidic, heavy metal-contaminated habitat, and microalgae occurrence

518 and diversity in this acidic river have been well studied. Genera of the phylum
519 Chlorophyta, such as *Chlamydomonas, Chlorella*, or *Dunaliella* have been detected in
520 the Tinto River (Zettler et al., 2002; Aguilera, 2013). Some examples of these genera
521 have been reported to possess high metal tolerance (León-Vaz et al., 2021) and many
522 of them have shown their ability to easily evolve into acidic and heavy metal adapted
523 species (Zettler et al., 2002).

524 Diatoms of the phylum Ochrophyta, such as the genus *Pinnularia*, have been previously
525 reported to be present in the Rio Tinto waters (Aguilera, 2013), however, we have not
526 found this genus in our survey. The most abundant Ochrophyta representatives in the
527 piezometer are the genera *Poteriospumella* (1.1%), *Nanochlorpsis* (1.4%), and an
528 uncertain genus of the family Chromulinales (3.5%). Whithin the Dinoflagellata phylum,
529 the genera *Gyrodinium* (7.7%) and *Alexandrium* (0.4%) are the most represented in the
530 perimeter channel, and *Alexandrium* (1.1%) the most abundant in the piezometer. This
531 last genus is also significant in the seawater (8% of total eukaryotic OTUs). Both
532 dinoflagellates have widespread marine distribution. Some species of the genus
533 *Alexandrium* are known as usual components of toxic algal blooms, but no acidophilic or
534 acidotolerant species of this genus have been described so far. Other non-
535 photosynthetic eukaryotes, which do not belong to the group of the fungi, have appeared
536 also in our study of the Huelva PG. The phylum Ciliophora is found in the perimeter
537 channel (12.3%) and in the piezometer (2.2%) at a high percentage. The phylum
538 Cercozoa, on the other hand, is present at percentages of 1.2 and 0.2% in the piezometer
539 and the perimeter channel, respectively. The sequences retrieved in this study share a
540 high percentage of identity with other uncultured ciliates and ameboflagellates, however,
541 there is not enough information for their assignation to a specific genus. Previous studies
542 describe the existence of ciliates, amoebas and other protist predators in the Tinto River
543 (Zettler et al. 2002).

Additionally, traces of other eukaryotes, including, arthropods, nematodes, higher plants, or rotifers have been detected in the PG waters. Monogonontos, a sub-clase of rotifers that includes some acidophilic species, is an example present at a considerable percentage in the piezometer water (4%), but not in the perimeter channel. This rotifer class was discovered in highly acidic mining lakes (Ersabek et al., 2011).

To our knowledge, there has not been any previous description of the eukaryotic community able to live at PG sites in any world location. However, there are several studies that report the presence of eukaryotic extremophiles in acidic volcanic environments (Russo et al 2008) or acidic mine drainages sites (Gadanho et al., 2006). One of the best-studied acidic environments is the Rio Tinto, where an unexpectedly high diversity of eukaryotes was found (Zettler et al., 2002; Aguilera, 2013). However, differently from what was observed in the bacterial profile, the eukaryotic community found in the PG differs significantly from that inhabiting the acidic mine drainage waters. It seems that not all the eukaryotes able to dominate the extremely acidic, heavy-metal-contaminated mine environments are able to tolerate the additional harsh conditions of the PG water or the even lower pH values.

The influence of the seawater in the piezometer sample is clear, not only because of its physicochemical parameters, but also due to the high percentage of marine microalgae and a much lower representation of freshwater fungi and ciliates, which are the main eukaryotic group in the perimeter channel water.

3.5. Functional modelling analysis

This research reveals the extraordinary variety of prokaryotic and eukaryotic microorganisms inhabiting the leachates obtained from Huelva PG. This population includes autotrophs and heterotrophs which assimilate inorganic or organic carbon compounds, respectively. Includes aerobic species which use oxygen as the final

570 acceptor of electrons in the respiratory electronic chain, and a series of versatile
571 anaerobic species able to replace oxygen by ferric iron or other oxidized species as
572 electron acceptors. Many acidophilic bacteria are facultative anaerobic that can adapt
573 their respiratory metabolism to diverse environmental conditions or use the reduction of
574 ferric iron to obtain extra energy. Regarding the autotrophs, besides the oxygenic
575 photosynthetic microalgae, a series of acidic chemolitotrophes able to fix CO_2 and obtain
576 energy from the oxidation of ferrous iron, sulfide, or other reduced sulfur compounds,
577 have been found in this study. Fig. 4 schematically represents the main pathways of this
578 complex panorama, citing some of the main representatives of each metabolic group.

579 To obtain further insight into the functional characteristics of this microbial population the
580 sequences retrieved from the metaxonomic study on the basis of the 16S rRNA marker
581 gene were analyzed with the use of the PICRUSt2 software and the functional gene
582 databases Kyoto Encyclopedia of Genes and Genomes (KEGG) or *MetaCyc.* This
583 analysis has allowed us to predict the abundance of gene families and obtain ontology
584 pathway predictions in the studied PG leachates and in the seawater or the Tinto River
585 samples (Fig. S2)

586 For both, the perimeter channel and the piezometer samples, the main functions
587 predicted using the PICRUSt algorithm can be classified within the categories: Metabolic
588 pathways, Genetic information processing, Environmental information processing, and
589 Cellular processes. A more detailed insight within the most represented category reveals
590 that the carbohydrate metabolism, with 900 or 912 orthologous genes (OG) in the PZ
591 and the PC respectively, the amino acid metabolism, with 613 and 620 OG in the PZ and
592 the PC respectively, or the energy metabolism with 473 and 500 OG in the PZ and the
593 PC, respectively, are the most represented metabolic pathways (Fig. S2A). Among the
594 most represented metabolic routes in the PG leachates, as well as in seawater and the
595 Tinto River, those related to methane metabolism, oxidative phosphorylation or amino
596 sugar, and nucleotide sugar metabolism stand out (Fig. S2B).

Comparative functional analysis between orthologous genes in the two studied PG leachates shows that there are 5937 common orthologous groups, while 292 and 346 are unique for the PZ and the PC, respectively. Regarding the functional categories, about 389 had representation in both PG leachates and only 11 of them were exclusively found in the PZ or the PC. Many of these genes and functional pathways are also found in the Tinto River and the seawater, although there are two functional pathways from PC the and one from the PZ which are unique to these locations, and have been found neither in the seawater nor in the Tinto River, such as the biosynthesis of mannosylglycerate (PWY-5656) or the degradation of creatine (PWY-4722) are exclusively found in the PZ or the PC, but not in the seawater or the Tinto River (Fig.5). Mannosylglycerate (MG) is an osmoregulatory solute typical of some halophilic organisms and creatine is a natural amino acid that can act as a source of nitrogen and carbon under certain circumstances. The presence of these pathways can be related to the existence of extreme conditions and low content of nutrients.

4. CONCLUSIONS

The leachates released from the PG stacks of Huelva constitute a unique environment of extreme acidity and high concentration of heavy metals and radionuclides, which has not been characterized from the microbiological point of view although these microorganisms can play an important role in the dynamics of the polluting compounds of the PG. Our study demonstrates for the first time that, in spite of the extreme physicochemical conditions, a high diversity of prokaryotic and eukaryotic microorganisms can thrive in the two locations studied, the perimeter channel that surrounds the PG stacks and the underground water collected in the piezometers settled at the borders of the stacks.

The prokaryotic profile resulted to be quite similar in the two leachates studied. The most abundant bacteria correspond to acidophilic genera, usually found in environments influenced by AMD, such as the Tinto River. Chlorophyte microalgae and Basidiomycota fungi, are the most abundant eukaryotic groups. This metabarcoding survey has also made clear the practically inexistence of representatives of the domain Archaea, as well as the null existence of species from the prokaryotic group of photosynthetic cyanobacteria or from the group of the sulfate-reducing bacteria.

Acidic and oxidizing conditions are more extreme in the perimeter channel than in the piezometer, where conductivity and salinity are higher, confirming the higher influence of the seawater in this sample. The influence of seawater in the PZ is also evidenced in the composition of the eukaryotic community. While the eukaryotic population of the PC water is dominated by the fungi, which represents 47% of the eukaryotes, the PZ has a much more important contribution of marine microalgae, which reach 62% of the total eukaryotes.

This study shows that the high content of pollutants of the PG influences the microbial diversity in the leachates. The information about the composition of the microbial community of the PG can shed light on the possible role that these microorganisms play in the dynamics of the contaminating compounds of the PG and can contribute to the obtaining of new strains with biotechnological applications.

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651 **Figure captions**

652

653 **Fig.1.** Map of the Huelva phosphogypsum stacks with definition of the different
654 zones and location of the sampling points: piezometer (PZ) and perimeter
655 channel (PC).

656

657 **Fig. 2.** Relative abundance of prokaryotic OTUs at various taxonomic levels.
658 Perimeter channel (PC), piezometer (PZ), sea water (SW) and Tinto River (TR)

659

660 **Fig. 3**. Relative abundance of eukaryotic OTUs at various taxonomic levels

661

662 **Fig. 4.** Main metabolic processes occurring in the PG leachates. Some significant
663 representative species are included. Light grey metabolic pathways and /or
664 species have not been found in these samples. See the text for a more complete
665 description

666

667 **Fig. 5.** Venn diagrams of the number of shared and unique functionally annotated
668 orthologous genes (A) and metabolic pathways (B) in the perimeter channel (PC),
669 the piezometer (PZ), the sea water (SW) and the Tinto River (TR) obtained using
670 MetaCyc database for funtional comparision. (C) MetaCyc codes for unique
671 pathways in each location

672

673

674 **Supplementary material**

675

676 **Table S1.** Primer sequences used to target the corresponding hypervariable
677 regions for the construction of the libraries.

678 **Fig. S1.** Rarefaction curves. The Observed features and the Shannon index
679 values for the 16S and the 18S data are plotted against the sequencing depth,
680 which referees to the product of the number of sequences by their length in
681 nucleotides.

682

683 **Fig. S2.** KEGG Mapper Reconstruction Result. The number of functionally
684 annotated genes (K terms) for each of category of pathway (A) and for the
685 majoritary metabolic pathaways (B) obtained from Picrust analysis
686 (https://github.com/picrust/picrust2) of the piezometer (PZ), the perimeter cannel
687 (PC), the seawater (SW) and Tinto River (TR) samples is shown

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693 **Table 1.** Physicochemical parameters, concentration of the most significant
694 elements and ions (mg L^{-1}), and activity concentration (Bq L^{-1}) of the main
695 radionuclides in the water samples collected from the perimeter channel (PC) and
696 the piezometer (PZ) at the PG stacks of Huelva (SW, Spain). Values for the
697 seawater (close to Huelva City) and Tinto River (near Niebla Town) from previous
698 studies have been included for comparison.

699

Variable	PC	PZ	Seawater[a]	Tinto River[b]
pH	1.56	1.7	7.78	2.7
T (ºC)	25.5	21	12.8	-
Eh (mV)	674	480	465	689
EC (mS cm^{-1})	41.8	53.6	61.5	2.4
As	32.8	9.2	<0.002	0.224
Cd	9.81	2.5	<0.002	0.089
Co	0.8	0.2	<0.002	0.54
Cu	9.8	3.1	0.0035	18.9
Fe	104	34.9	0.0081	144
Hg	<0.01	<0.01	-	<0.01
Mn	14.5	7.6	<0.002	8.6
Ni	6.3	1.8	<0.002	0.14
Pb	0.5	0.1	0.003	0.097
S	1722	1246	1040	1550
Sb	0.9	0.11	0.011	<0.1
Se	<0.1	<0.1	-	<0.1
Zn	74.3	20.8	0.050	20.7
Ca	1250	832	441	82
K	459	561	440	5.1
Mg	667	730	1440	85
Na	4027	9536	11700	46
F^-	1377	85	111	2.9
Cl^-	6778	16619	23563	72
SO_4^{2-}	17077	3231	3288	4810
^{238}U	302	24	0.042	0.057
^{234}U	302	23	0.045	0.112
^{230}Th	12	0.62	<0.0001	0.044
^{232}Th	0.12	0.03	<0.0001	0.023
^{226}Ra	0.73	0.62	-	-
^{210}Po	22	43	0.0036	0.011

700 [a]Guerrero et al., 2021a. [b]Guerrero et al., 2021b

701

Table 2. Sequence data statistics

Sample	Raw Reads	After denoising	Merged inputs	Mean quality Q_{30} (%)	Mean quality Q Score	Observed OTUs	Shannon Index
PC_16S	19129	14995	10555	94.60	≥ 36	680	7.7
PC_18S	10067	8864	6076	93.36	≥ 36	38	1.5
PZ_16S	17129	13236	9409	94.54	≥ 36	596	7.5
PZ_18S	30362	21890	15080	89.74	≥ 36	186	4.5
TR_16S	189164	175352	145395	95.12	≥ 36	348	5.1
TR_18S	160136	126280	103365	90.99	≥ 36	133	6.3
SW_16S	206161	190181	171677	95.17	≥ 36	838	6.4
SW_18S	203677	172627	111906	92.24	≥ 36	399	4.5

The number of raw sequences, sequences after denoising, non-chimeric merged inputs, and Observed Operational Taxonomic Units (OTUs) obtained for each sample are shown. The mean quality score (Q score), the percentage of sequences with a Q score higher than 30 (Q30) and the Shannon biodiversity index for each sequencing run have also been included. Sample names: PC, perimeter channel; PZ, piezometer; TR, Tinto River; SW, Sea water.

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Figure 1

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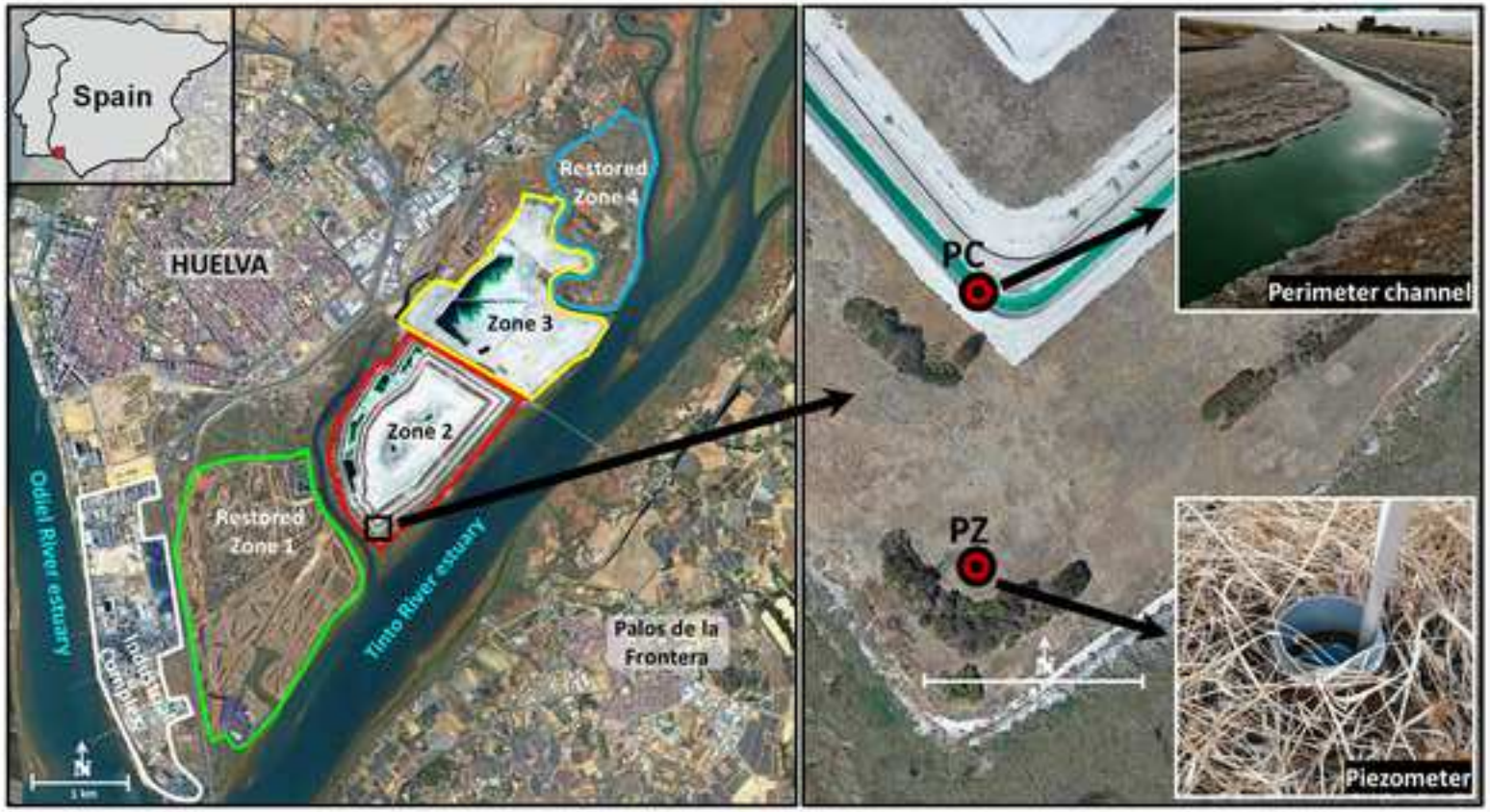

Figure 2

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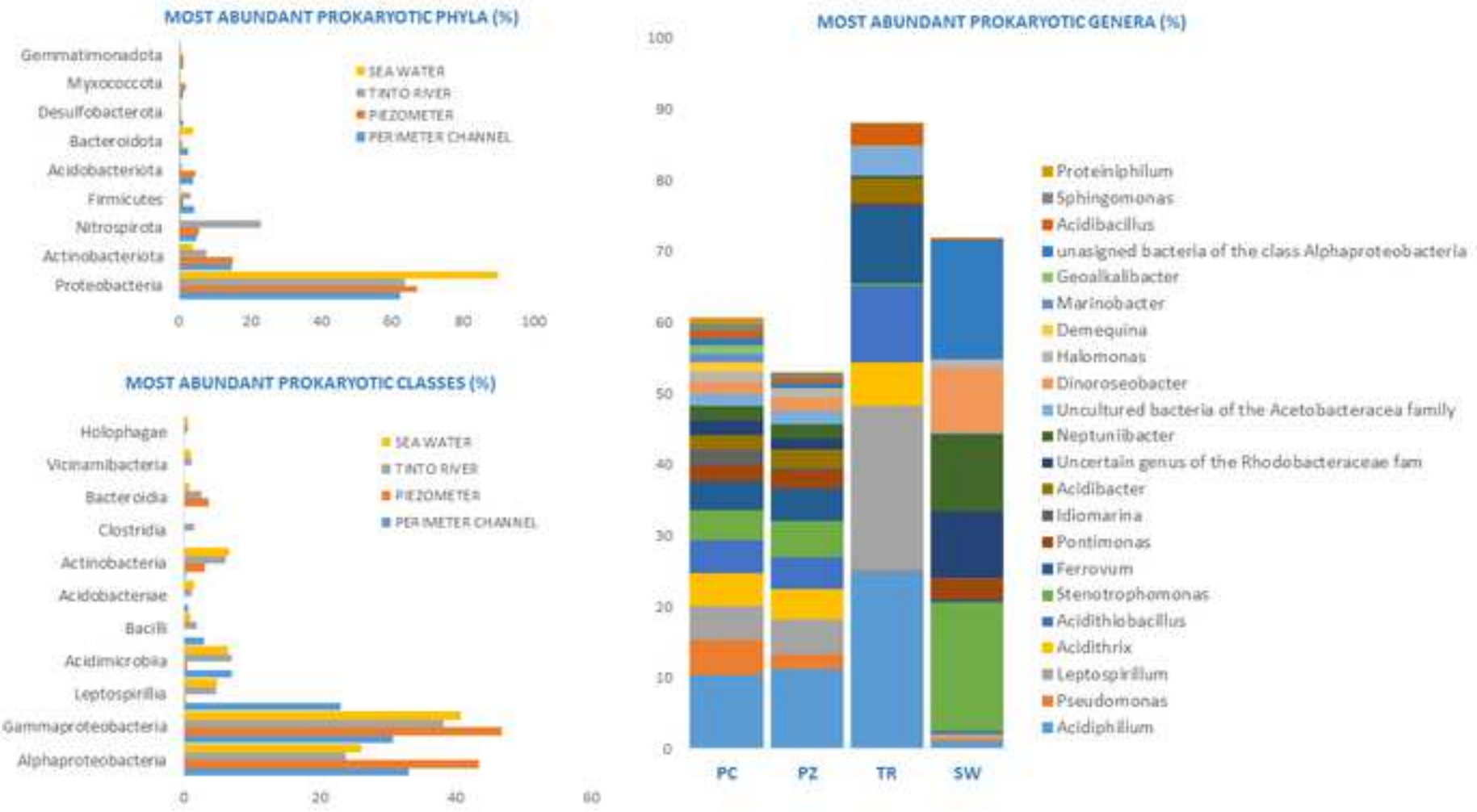

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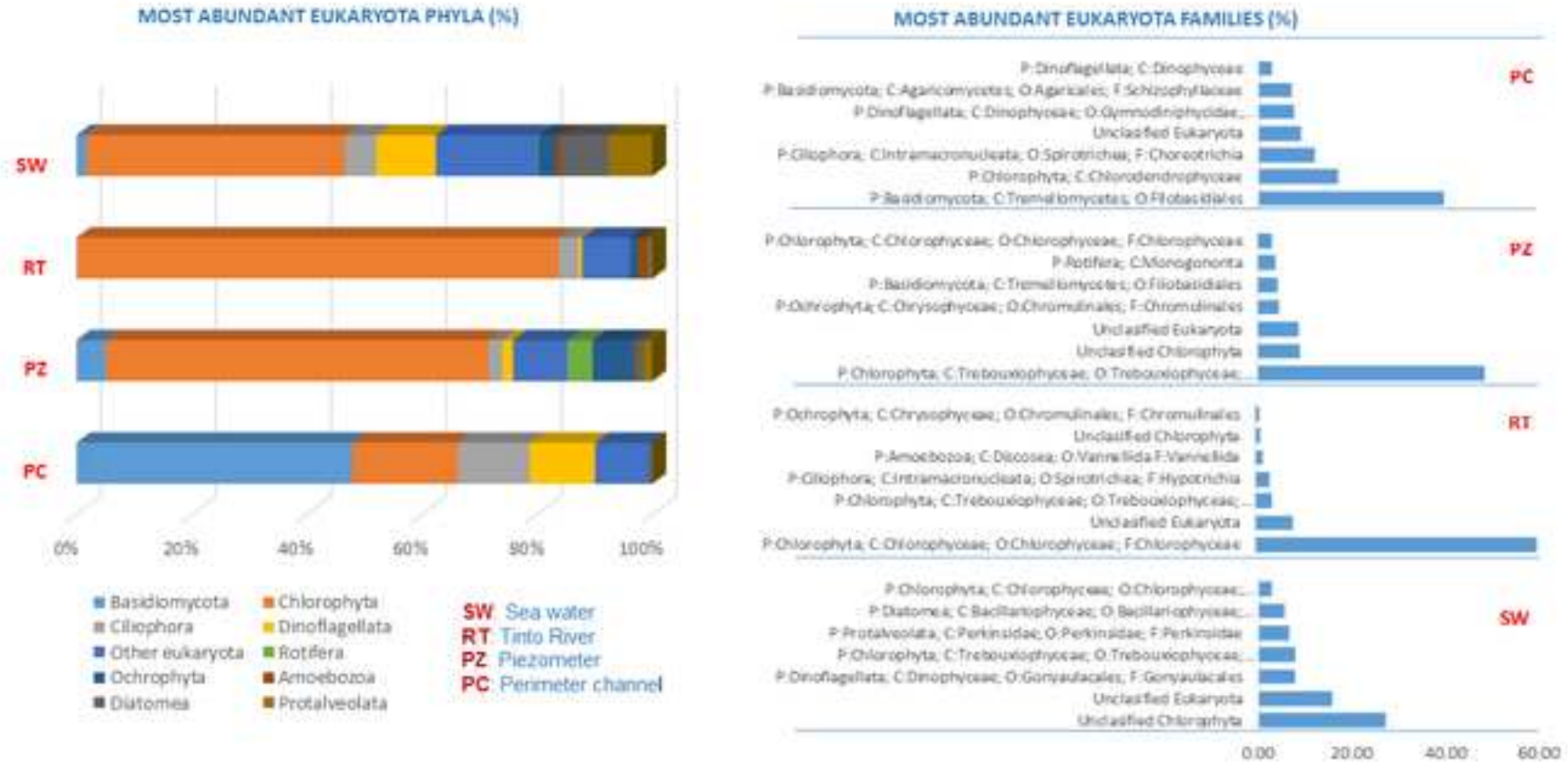

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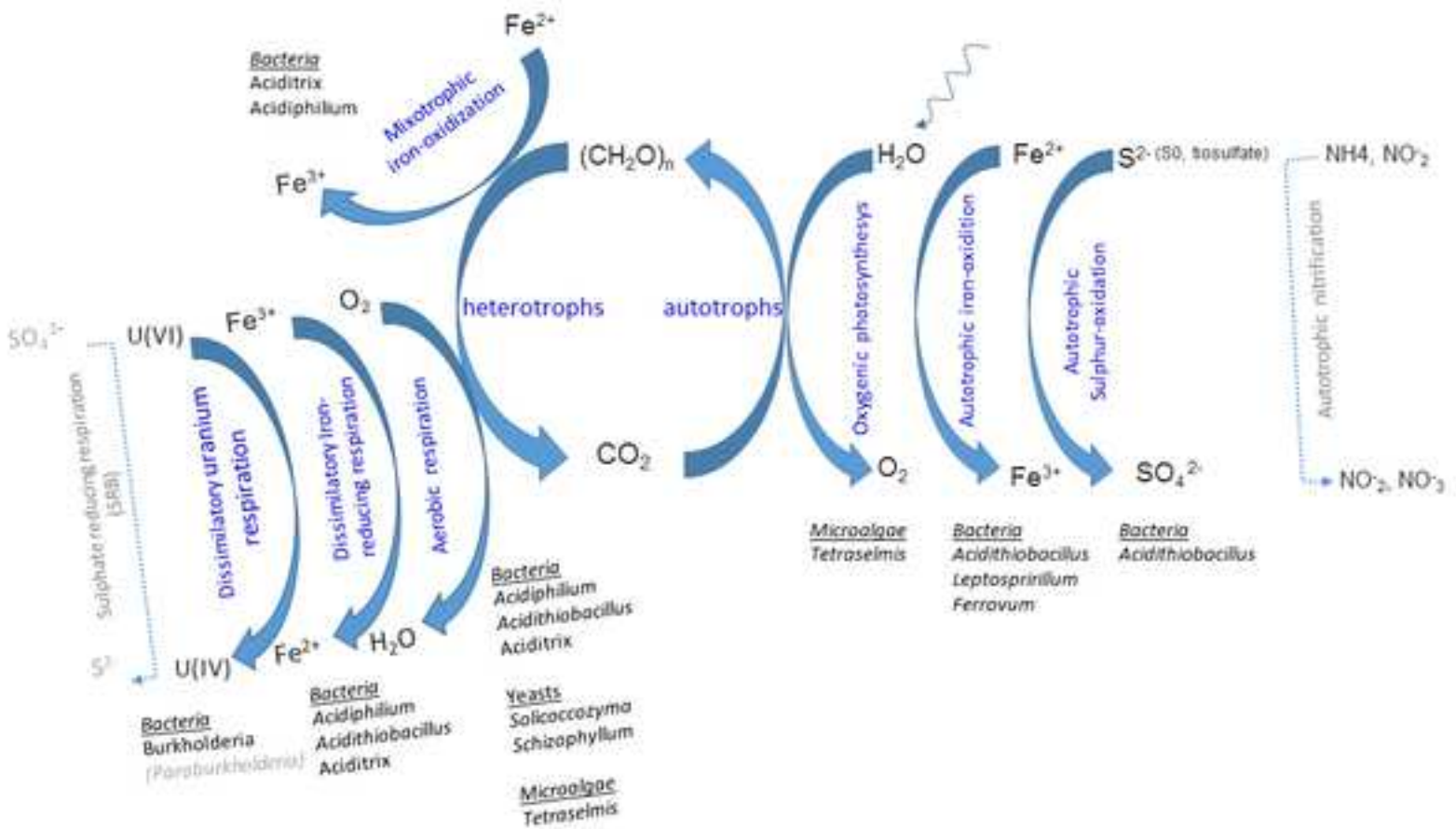

Figure 5

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