Prokaryotic diversity in stream sediments affected by acid mine drainage

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Abstract

The microbial communities in mining impacted areas rely on a variety of mechanisms to survive in such extreme environments. In this work, a meta-taxonomic approach using 16S rRNA gene sequences was used to investigate the prokaryotic diversity of sediment samples from water bodies affected by acid mine drainage at the São Domingos mining area in the south of Portugal. Samples were collected in summer and winter from the most contaminated sites from where the water flows downstream to the freshwater of Chança's river reservoir. The prokaryotic diversity on water bodies' sediments allowed us to distinguish the highly contaminated sites (pH ≈ 2) from sites with intermediate levels of contamination (pH ≈ 3 to 6.5), and from sites without contamination (pH ≈ 7.5). The abundances of acidophiles of genera *Acidiphilium, Acidibacter, Acidobacterium and Acidocella* in the sediments were correlated with the level of acid mine drainage contamination. The two first genera were among the 30 most abundant prokaryotes in all contaminated samples, including one (SS2w) where the contamination was very diluted, thereby emphasizing the impact that such type of pollution can have in the microbial communities of sediments. In addition, the high abundances of archaeal taxa from class *Thermoplasmata* and of bacteria from family RCP1-48 in the sediments from the most contaminated site corroborate their importance in such ecosystems and a putative role in the generation of acid mine drainage.

Key words: acid mine drainage, metal contamination, microbiome, sediments.

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1. Introduction

The exploration of minerals has been for many centuries one of the most important human activities in the world. However, this mineral activity can become a source of contamination, causing major environmental problems such as the generation acid mine drainage (AMD). Also known as acid rock drainage, AMD is a strong acidic wastewater with high concentrations of sulphates, metals and metalloids which can contaminate ground and watercourses, damaging the health of aquatic species, plants, wildlife and humans (Johnson 2003; Simate and Ndlovu 2014). The main cause of AMD is the oxidation of sulphide minerals (mainly pyrite) resulting from their exposure to oxygen, water and microorganisms, which may occur naturally but is usually accelerated by mining activities that increase the exposure of those minerals (Johnson 2003; Johnson and Hallberg 2005; Egiebor and Oni 2007).

São Domingos mine is one of the most important inactive mines in Portugal. It is located in the Beja district of Lower Alentejo region of southern Portugal (37°40'08"N 007°29'38"W) in the Iberian Pyrite Belt, which is one of the largest metallogenetic provinces of volcanogenic massive sulphides in the world (Álvarez-Valero et al. 2008; Alvarenga et al. 2012). Massive pyrite, an iron sulfide (FeS_2), was the main mineral ore extracted in the mine, but extraction also included ores associated to copper (Cu), aluminium (Al), arsenic (As), lead (Pb), antimony (Sb), zinc (Zn) and mercury (Hg) (Tavares et al. 2008). This mine was originally exploited during the Roman and Islamic occupations of the Iberian Peninsula, but from 1857 to 1966 it was intensively active. However, since the mining activity ended (1966) the facilities were abandoned and the mining area has not been properly protected or monitored. Intensive mining activity has produced considerable amounts of residues, and consequentially, a serious environmental deterioration of the local environment (Alvarenga et al. 2012; Dias-Sardinha et al. 2013). A highly acidic water stream is present and contaminates downstream water bodies in the area. It flows from the open pit zone and passes through a large area of mining debris until it joins the Mosteirão stream and then meets with freshwater from Chança´s reservoir (Ettamimi et al. 2019).

Several studies have determined the concentrations of toxic trace elements (As, Sb, Pb, Cu and Zn) in sediment, water and plants in the São Domingos area in order to evaluate the environmental impact of the mine (Alvarenga et al. 2012). The highest metal concentrations, especially of Al, Fe, Cu, Zn and Mn, together with the lowest pH values (pH = 2.3–3.1) were documented during the dry season in water bodies samples close to the mine

area (e.g. Ettamimi et al. 2019). Recently the prokaryotic communities of water bodies contaminated by AMD at the São Domingos mining area were studied through a meta-taxonomic analysis (Ettamimi et al. 2019). In the study it was shown that the most contaminated sampling sites (pH = 2.3–3.1) were distinguished by a lower prokaryotic diversity and a high abundance of acidophiles, while in the transition zone at the mouth of the contaminated water flow into the Chança´s reservoir (pH = 6.4), a specific prokaryotic community exists with some acidophiles, but notably with a cyanobacteria bloom and a high abundance of the genus *Sediminibacterium* (Ettamimi et al. 2019). The application of meta-taxonomic methods based on a total extraction of DNA directly from environmental samples enables access to the unknown portion of the microbial communities that remain inaccessible when using classical methods of cultivation and identification (Jovel et al. 2016).

The main objective of this work was to study the prokaryotic communities in the sediments of water bodies from the São Domingos mining area through a meta-taxonomic approach using 16S rRNA gene amplicons. In addition, the correlation between the most abundant prokaryotes of the studied sediments and the physicochemical characteristics of the waters collected above them was analyzed to assess the effects of AMD pollution on the prokaryotic diversity of water bodies' sediments.

2. Material and methods

a. Sample collection and processing

Sediment samples from the superficial layer (2-10 cm deep) were collected using sterile falcon tubes (15 mL volume) at a water depth of about 0.5 m (measured with a 0.5m plastic stick) at different sites in the São Domingos mining area. The tube was manually (with sterile vinyl gloves on) submerged with the opening facing down to minimize entry of water and completely buried in the sediments together with the index finger, which was used to immediately cover the opening (still in the sediments). Then the tube filled with sediments and covered with the finger was removed from the water and closed with a cap. Approximately 15g of sediments from each site were collected, then immediately (~2 hours) transported on ice to the laboratory and kept frozen at -20ºC until processing for DNA extraction.

Each site was selected based on its distance to the pit lake and surrounding mine-waste piles, which are the main source of AMD contamination (Fig. ESM-1). Longitude and latitude for each site were defined using a Map 330 GPS. The sampling sites and sampled sediments designations, each site local name and coordinates, and their brief descriptions are summarized in Table 1. Site 0, located on the Mosteirão stream before it merges with the São Domingos stream was selected as a control sample. Site 2, located at the Chança reservoir, was chosen as it is likely a site where the AMD contamination is very low. Site 5 was selected due to its location in the area called "Telheiro" in the confluence of the contaminated São Domingos stream and the non-contaminated Mosteirão stream, while sites 3 and 6 were selected due to their locations downstream and upstream to the confluence point, respectively. Site 7, a small dam at São Domingos stream located downstream the dam called "Tapadinha", is highly impacted by AMD. Sampling was performed during winter and summer seasons, in February and September 2017, respectively, aiming to study the impact of seasonal factors on prokaryotic diversity of the sediment samples. However, the sites S0, S5 and S6 were dry in summer and, thus, were not sampled. That year (of extreme drought) it rained truly little in the south of Portugal, even in winter. The streams of the São Domingos mining region dried up completely in the summer; there was water only in the rivers and dams. Therefore, the samples taken in the summer for this study are few, but they represent what existed. This was not totally unexpected since the climate in this region is semiarid mesothermic with hot and dry seasons extended throughout a long part of the year (Quental et al. 2011). Regarding the outsider control, since the supply of AMD to the Chança reservoir was interrupted due to drought, the sediment sample collected in that reservoir in the summer (SS2s) ended up serving that purpose.

It is known that both the water quality and the sediments properties have influence on the species composition, diversity, and abundance of sediment microbial communities in aquatic systems (e.g. Freixa et al. 2016; Jiang et al. 2017). Surface waters sampled at all sites just before sampling the sediments were previously analyzed to evaluate the level of AMD contamination and prokaryotic diversity in the water (Ettamimi et al. 2019). The focus of this study is to evaluate the impact of different levels of AMD pollution in waterbodies (water quality factors) on the prokaryotic communities present in the surface layers of their sediments. Therefore, the characterization of the surface waters previously carried out by Ettamimi et al. (2019) at the sampling sites (Table 2) is used to evaluate putative correlations

between the physicochemical characteristics of those waters and the prokaryotic communities on the superficial layer of sediments sampled.

b. DNA extraction

A sediment sample from each sampling site was homogenized and subjected to DNA extraction using the PowerSoil DNA Isolation Kit (MO BIO) as described in the protocol. The concentration and quality of eluted DNA was determined using a spectrophotometer (NanoDrop3300, Thermo Fisher Scientific, USA).

c. 16S rRNA amplicon library preparation and sequencing

Purified DNA extracted was used for single-end library construction based on an Illumina protocol (Illumina, 2015). The V4 regions of the 16S rRNA genes were amplified by polymerase chain reaction (PCR) using (515F) and (806R) primers (5'-GTGCCAGCMGCCGCGGTAA and 5'-GGACTACHVGGGTWTCTAAT). The forward and reverse primers were designed specifically for prokaryotic 16S gene V4 region (Caporaso et al. 2011). PCR reactions of 25 µL were performed with up to 10 ng of extracted DNA as template, 400 nM of each forward and reverse primer, 100 µM of each dNTP, 1.5 mM of $MgSO_4$ and 1 U of Platinum®Taq DNA polymerase HF, in 1X Platinum® High Fidelity buffer (Thermo Fisher Scientific, USA). PCR cycling was conducted under the following program: initial denaturation at 95°C for 2 min, 35 cycles of amplification (95°C for 20 s, 50°C for 30 s, 72°C for 60 s) and a final elongation at 72°C for 5 min. Duplicate PCR reactions were performed for each sample and the duplicates were pooled after PCR. The amplicon libraries were purified using the standard protocol for Agencourt Ampure XP Bead (Beckman Coulter, USA) with a modified bead to sample ratio of 4:5, then eluted in 33 µL of nuclease-free water (Qiagen, Germany) and their DNA concentration measured using Qubit™ HS DNA Assay kit (Thermo Fisher Scientific, USA). Sequencing was performed by DNASense Company (Aalborg, Denmark). The sequencing libraries were prepared from the purified amplicon libraries using a second PCR. Each PCR reaction (25 µL) contained 1x PCRBIO HiFi buffer (PCRBiosystems, UK), PCRBIO HiFi Polymerase (1U) (PCRBiosystems, UK), adaptor mix (400 nM of each forward and reverse) and up to 10 ng of amplicon library template. PCR was run with following program: initial denaturation at 95°C for 2 min, 8 cycles of amplification (95°C for 20 s, 55°C for 30 s, 72°C for 60 s) and a final elongation at 72°C for 5 min. The resulting sequencing libraries were purified, eluted and quantified as described before for the amplicon

libraries. Finally, the purified sequencing libraries were pooled in equimolar concentrations and diluted to 2 nM, and the samples were sequenced (301bp) on a MiSeq (Illumina) using a MiSeq Reagent kit v3 (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. The Phix control library was used as an in-run control (20% spikedin) for run quality monitoring to overcome the issue of low complexity that is often observed with amplicon samples. The sequences obtained in this work have been submitted to the Sequences Read Archive SRA database with accession number: PRJNA593038.

d. Bioinformatics and statistical analysis

Initial processing included screening and removing short and low-quality reads using the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and trimming the sequences to 225 bp using Trimmomatic (Bolger et al. 2014). The reads were de-replicated and formatted using the UPARSE workflow (Edgar 2013), then the de-replicated reads were clustered into operational taxonomic units (OTUs) using the "cluster_otus" command of USEARCH (vers. 7.0.1090; Edgar 2013) with default settings. The OTU abundances were estimated for a 97% sequence identity using the "usearch_global" command with parameter-id 0.97. The taxonomic classifications of OTUs were assigned using the RDP classifier (vers. 11; Wang et al. 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (vers. 1.7.0; Caporaso et al. 2010), using the MiDAS database (vers.2.1.2; Mcilroy et al. 2017) and the results were analyzed in R (vers. 1.0.153; R Core Team 2017) through the RStudio IDE using the "ampvis" package (vers. 2.2.6; Albertsen et al. 2015). The prokaryotic diversity was studied using Shannon and Simpson diversity indexes, which are both calculated using the number of OTUs and respective abundances, thus considering the richness and evenness of OTUs. The former is more influenced by species richness and by rare species, while the second gives more weight to evenness and common species (Shannon 1948; Simpson 1949; Spellerberg and Fedor 2003; https://www.davidzeleny.net/anadat-r/doku.php/en:div-ind). These indexes were calculated using raw OTU tables and normalized OTUs tables since normalization (number of reads in each sample randomly equalized) in some datasets can distort alpha diversity comparisons. The relationships between samples studied using principal components analysis (PCA) in the "ampvis" package were based on OTU abundances higher than 0.1%, with data plotting focused on the two first principal components. In

addition, Shannon and Simpson diversity indexes were calculated with raw OTUs tables and with normalized OTUs tables, using the R package "RAM" (Chen et al. 2018).

3. Results and discussion

a. Prokaryotic community richness and evenness

A total of 244,261 single-end Illumina reads (225 bp after quality control) of the 16S rRNA gene's V4 region were obtained from sediment samples of the São Domingos mine. Table ESM-1 in the Online Resource shows the number of reads and OTUs obtained in each sediment sample.

Both the Shannon (Sh.) and Simpson (Si.) diversity indexes, calculated using raw or normalized OTU tables, revealed similar results (Table 3). These indexes have greater values in all sampled sediments (Sh. = 2.753 to 6.311; Si. = 0.839 to 0.996), comparing with those in the waters sampled above them (Sh. = 2.112 to 4.673; Si. = 0.823 to 0.977) previously analyzed by Ettamimi et al. (2019). The most probable cause for this higher prokaryotic diversity in the sediments than in the waters above them is that in the water the physicochemical conditions are homogeneous, while in the sediments micro-niches can be generated with different physicochemical conditions, favoring optimal conditions for more diverse microbes. Indeed, differentiated micro-layers of minerals in the bed of waterbodies and the accumulation of organics that fall to the bottom may cause the establishment of stratified microbial communities in the sediments nearby the water-sediment interface due to stratified physicochemical conditions (for example different layers of oxic conditions).

In the sediments, the indexes indicate a decreasing gradient of diversity from the lowest to the highest AMD impacted waterbodies. Indeed, the indexes were much lower in the sediments from the most contaminated site S7, the closest to the mine open pit and mine debris (sample SS7w: Sh. = 4.415, Si. = 0.977 and sample SS7s: Sh. = 2.753, Si. = 0.839) than in the sediments from sites with low or intermediate levels of AMD impact (samples SS2w, SS3w, SS5w, SS6w, SS2s and SS3s: Sh. = 5.673 to 6.311, Si. = 0.989 to 0.996). Notably, the indexes in the sediments from the reference water stream not affected by AMD (sample SS0w: Sh. = 4.988, Si. = 0.982) indicate a lower diversity compared to the sediments from sites with low or intermediate AMD impact. These results indicate that the impact of AMD in water bodies until a certain level causes a slight raise of prokaryotic richness and evenness in the sediments,

but after a certain degree of higher contamination it causes a clear decay of both these facets of diversity.

b. Prokaryotic community composition

Figure 1 shows the relative abundances of dominant prokaryotic phyla (≥ 1% of the OTUs) in each sediment sample as classified using the V4 region of the 16S rRNA gene sequences. The 20 different phyla with such high relative abundances in all sediments analyzed in this work compared with the 11 phyla in the water samples previously studied (Ettamimi et al. 2019), confirm the highest diversity in sediments revealed by the Shannon and Simpson indexes.

Comparing all sediments reveals a decreasing trend in the number of phyla from the non- or less-contaminated sites to the highest contaminated sites (Fig. 1). At this high taxonomic level, SS0w was the most diverse sediment sample with thirteen phyla detected plus the groups of "others" and "unclassified". Samples SS6w, SS7w and SS7s, from the highest contaminated sites, were the least diverse with only 5 or 6 phyla identified plus the groups of "others" and the "unclassified". In these sediment samples the common most abundant phyla were *Proteobacteria, Acidobacteria*, *Actinobacteria* and *Planctomycetes*. The first three are usually also among the most abundant phyla in sediment samples from water bodies with similar levels of AMD impact in other mining areas, and the last is referred just in some cases (Zhang et al. 2019; Sanchez-Andrea et al. 2011; Mesa et al. 2017; Korzhenkov et al. 2019).

Apart from these four phyla, other two were highly represented just in sample SS6w: *Bacteroidetes* and *Verrucomicrobia*; while another two were among the most abundant just in sample SS7s: *Nitrospirae* and *Euryarchaeota*. Three of these phyla were also highly represented in sediments from contaminated sites, depending on the studied mine; for example: *Bacteroidetes* (Zhang et al. 2019), *Euryarchaeota* (Korzhenkov et al. 2019), and *Nitrospirae* (Mesa et al. 2017). By contrast, *Verrucomicrobia* is usually detected with relatively high abundances in freshwater reservoirs (eg. Llirós et al. 2014) and indeed, this phylum was common in the reference samples of sediment or water from water bodies not contaminated with AMD studied at the mining areas (eg. Ettamimi et al. 2019; Zhang et al. 2019). Thus, the relatively high abundance of *Verrucomicrobia* in sample SS6w could be due to the merging of uncontaminated water streams with the AMD flow that occurs in some points between the mine and the sampling site S6.

The prokaryotic diversity was also studied at lower taxonomic levels for a better understanding of the differences between taxonomic profiles on the sediments. Tables ESM-2 and ESM-3 represent the 30 most abundant prokaryotes among all sites sampled in winter and in summer. In winter, there were five taxa (first five entries of Table ESM-2) present in relatively high abundances in the sediments of the highest contaminated site (SS7w), while they were less abundant or nearly absent in samples with less AMD contamination (SS2w, SS3w and SS5w) and absent in the sediments from the non-contaminated site (SS0w). In contrast, the sediments from the water stream not affected by AMD had a clear and specific taxonomic profile comparing to the other sediments with lower or higher AMD impact. Five taxa (entries 16, 17, 18, 20 and 27 of Table ESM-2) were only present in sample SS0w, while the other taxa (remaining entries of Table ESM-2) were mainly present in the sediments from the sites with low or intermediate levels of AMD impact (SS2w, SS3w and SS5w). A similar trend was even more evident in summer when eight taxa (the first eight entries of the Table ESM-3) were present in the sediments of the highly contaminated site (SS7s) and absent in the sediments of the other two sites (SS2s and SS3s) and the opposite was observed for the other 22 taxa (remaining entries of Table ESM-3).

Taxa from *Acidibacter* genus and *Acidobacteriaceae* family (Subgroup 1), including *Acidobacterium* genus, had relatively high abundances in the sediments from the most contaminated site either in winter (SS7w) or in summer (SS7s), and were also among the 30 most abundant taxa in sediments with lower levels of AMD impact, but were not detected in the sediments from the non-contaminated water stream, suggesting a strong influence of AMD impact on the sediments' microbiomes even for sites with low contamination. *Acidibacter* can be particularly interesting because it was among the most abundant taxa in all sediments with AMD contamination, even when the contamination was so diluted (sample SS2w) that could go unnoticed by quick on-site measurements of physicochemical parameters.

Genera *Acidiphilium* and *Acidocella* were in evidence in the sediments from the most contaminated site in winter; specially the first that was also among the most abundant prokaryotes in all contaminated winter sediments, including sample SS2w. The genera *Leptospirillum* and *Thermoplasma*, the families "RCP1-48" and *Ferroplasmaceae* and the order

"CPla − 3 termite group", stood out in the sediments from the most contaminated site in summer.

The genera *Acidibacter*, *Acidiphilium*, *Acidocella* and *Leptospirillum,* the *Acidobacteriaceae* family (Subgroup 1) and the order "CPla-3 termite group" had also been detected among most abundant taxa in the water samples collected at the same sites (Ettamimi et al. 2019). This was not unexpected since the studied sediments are from the superficial layers, including the sediment-water interface. Moreover, these taxa had already been identified in other acidic and metallic environments impacted by AMD (Barns et al. 1999; Hao et al. 2007; González-Toril et al. 2010; Ziegler et al. 2013; García-Moyano et al. 2015; Zhang et al. 2019).

The arhaeal *Thermoplasma* genus (9.1%) and *Ferroplasmaceae* family (4.6%) as well as the bacterial RCP1-48 family (36.4%) were detected among the most abundant taxa of contaminated sediments, but not in the contaminated waters previously analysed by Ettamimi et al. (2019). It is known that besides the importance of aquatic planktonic microorganisms, the microbial communities in the sediments also play a key role in the nutrients balances and cycling in aquatic ecosystems. Thus, these taxa may have important roles in the ecosystems of waterbodies affected by AMD.

Ferroplasmaceae have been commonly found in acidic mine tailings and have been associated to the AMD pollution (Ferrer et al. 2007; Chen et al. 2014). However, to our knowledge, it is the first time that the genus *Thermoplasma* specifically has been associated with this type of pollution. Both the genus *Thermoplasma* and the family *Ferroplasmaceae* are *Euryarchaeota* from *Thermoplasmata* class, which are all acidophiles and most thermophilic (Garrity et al. 2001), thus it makes sense that they were in evidence in the most acidic site (pH 2.3) in summer, when temperatures reach values over 40^{o}C. Moreover, in a recent study at the Parys Mountain copper mine (Anglesey, UK) with AMD characterized by extremely low pH (1.7) and high concentrations of soluble metal cations, new clades of *Thermoplasmata* class with yet unclear functional roles in the ecosystem had the highest abundances (Korzhenkov et al. 2019). Thus, it is becoming evident that several different taxa of this class can survive in waters strongly affected by AMD, probably having an important role in such ecosystems.

The family RCP1-48 of the order *Acidithiobacillales*, has also been detected in the sediments of water bodies affected by AMD at different mining areas, as for example the Rio Tinto

(Sánchez-Andrea et al. 2011) and the Los Rueldos mines (Mesa et al. 2017) of Spain. In fact, this family of bacteria is among the responsible for the presumably predominant metabolisms in the AMD microenvironments at Los Rueldos (Mendes-García et al. 2014) and it was suggested that its detection in high proportions on biofilm interfaces and in the most upstream acidic waters indicates they have roles in Fe/S oxidation, thus in the formation of AMD (Mesa et al. 2017).

c. Correlations between samples

The relationships between sediment samples in each season were analyzed using the Principal Component Analysis (PCA) based on the relative abundances of OTUs Fig. 2). The first two principal components, which accounted for 30.36% and 28.28% of the total variation in winter and 45.85% and 35% in summer, were calculated. Each point represents a sediment sample with more adjacent samples having more similar prokaryotic communities. Clustering of winter sediments based on the first two principal component separates them into three clusters: one group of sediments from sites with low to moderate contamination (SS2w, SS3w, SS5w and SS6w) which were separated from the non-contaminated sample SS0w, and from the most contaminated sample (SS7w). A similar clustering is observed with sediments collected in summer with samples SS2s and SS3s forming one group distinct from the most contaminated sample.

The focus of the study is to evaluate the impact of different levels of AMD pollution in waterbodies on the prokaryotic communities present in the surface layers of waterbodies' sediments. Therefore, although the physicochemical characteristics of the water may differ from those of the sediments, the correlation between the level of AMD pollution in the waterbodies and the prokaryotic communities in the surface layer of their sediments can be evaluated by comparing the PCA based on the waters' physicochemical characteristics (Fig. ESM-2) with the PCA based on the relative abundances of OTUs in the sediments (Fig. 2). This comparison reveals a distribution of sediment samples based on prokaryotic communities that is congruent with the distribution based on the physicochemical characteristics of the waterbodies from where the sediments were collected. This congruence suggests a strong influence of AMD pollution on the prokaryotic communities of water bodies' sediments.

d. Correlations between physicochemical characteristics of water bodies and most abundant prokaryotes in their sediments

To better understand the impact of AMD on the prokaryotic diversity of sediments, a Pearson correlation matrix was calculated between the relative abundances of the 30 most abundant taxa founded in the sediment samples and the physicochemical parameters of the water samples collected above them. These analyses were only performed for the winter season because in summer only three sites were sampled. On another hand, the temperature and dissolved oxygen (DO) parameters were excluded because they had little influence on the physicochemical discrimination of the water samples collected above the sediments (Ettamimi et al. 2019). The results are shown as clustering dendrograms and a heat map representing the "r" values of the correlation matrix (Fig. 3).

The dendrogram based on physicochemical parameters can be divided into two clusters: cluster-1, grouping the sulphate ($SO4^{2-}$) and the metals (Cu, Al, Fe, Zn) concentrations together with the EC, and cluster-2, which has the pH together with the concentration of phosphate (PO_4^{3-}).

By contrast, the dendrogram based on the 30 most abundant prokaryotes has four major clusters. From the top to the bottom of the heat map. Cluster-1 groups the first five taxa which show a strong positive correlation with cluster-1 of physicochemical parameters (metals, sulphate, and EC) and a negative correlation with cluster-2 (more evident with pH than with PO_4^{3-}). Cluster-2 is formed with the next 11 taxa which are clearly negatively correlated with physicochemical parameters of cluster-1 and with a weak negative correlation (or without) with one parameter of cluster-2 (PO_4^{3-}), while being positively correlated with the other parameter of cluster-2 (pH). Cluster-3 groups taxa in lines 18-25 which have no, or very weak positive or negative correlations with the physicochemical parameters of cluster-1 and negative correlations with the physicochemical parameters of cluster-2 (strong with pH and low to moderate with PO_4^{3-}). Cluster-4 which include the last five taxa have moderate negative correlations with parameters of cluster-1 and positive correlations with the physicochemical parameters of cluster-2. In addition, the genus *Telmatobacter* revealed a dissimilar aspect: very weak or no correlations with all physicochemical parameters, probably due to its presence in relatively high abundances in just two sediment samples from water bodies with

highly distinct physicochemical characteristics: 2.4% in SS2, 0.6% in SS7 and ≤ 0.1% in the other. This genus has bacteria able to degrade plant-derived biopolymers under micro-oxic or anoxic conditions (Pankratov et al. 2012) and therefore its abundance is more likely related to the presence of degrading plant tissue in the sampled sediments then with the level of AMD contamination. Nevertheless, this analysis allowed us to confirm the correlation between the impact of AMD contamination in the water bodies' sediments and the taxa identified in Table ESM-2. The abundancies of the first five most abundant taxa, namely, OTU_3 from class *Betaproteobacteria* (which was not possible to assign to a lower taxonomic level) and *Acidibacter, Acidobacterium, Acidocella,* and *Acidiphilium* genera, increase as the AMD contamination raises. The latter four genera were also among the taxa previously identified by Ettamimi et al. (2019) as being correlated with AMD contamination in the surface waters from the same sites studied in this work.

4. Conclusions

As a first meta-taxonomic study of sediment prokaryotes from water bodies in the São Domingos mining area, this work contributes to better understand the impact of AMD on these ecosystems. It reveals that differences in the prokaryotic communities of the water bodies' sediments can distinguish the sites that are highly contaminated with AMD (pH ≈ 2) from sites with intermediate levels of contamination (pH ≈ 3 to 6.5) and from sites without any AMD impact (pH ≈ 7.5). In addition, by comparison with a previous work, it is shown that the prokaryotic diversity richness and evenness in the sediments of such sediments is higher than in the waters above them. Finally, this work corroborates the importance of archaea from class *Thermoplasmata* and bacteria from family RCP1-48 to aquatic ecosystems highly affected by AMD and is one more evidence that they may have some role on the formation of this type of pollution.

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Captions

Figure 1. Prokaryotic phyla with high relative abundances (≥ 1%) in sediments from different sampling sites at the São Domingos mining area during (**a**) the winter and (**b**) the summer seasons. "Others" represents the sum of all taxa that scored relative abundancies below 1%.

Figure 2. Principal Component Analysis of taxonomic profiles with prokaryotic OTU abundances higher than 0.1%, in sediment samples collected at São Domingos mining area in (**a**) winter and (**b**) summer season.

Figure 3. Heat map and dendrograms showing correlations between physiochemical characteristics of the sampled water bodies at São Domingos mining area in winter and the 30 most abundant prokaryotic taxa in their sediments. Colors indicate R values correlations between physicochemical parameters (columns) and bacterial taxa (rows).

Table1. Location and description of the São Domingos water bodies sampled sites, and designation of sediments samples.

Table 2. Physicochemical characterization of waters above the sediments at sampled sites (adapted from Ettamimi et al. 2019).

Table 3. Shannon and Simpson diversity indexes calculated for all sediments samples with (**a**) non-normalized OTUs tables and (**b**) normalized OTUs tables, the pH of samples is also shown (in the first row). Colour scales were obtained by conditional formatting each row independently using the Excel software (Microsoft Office).

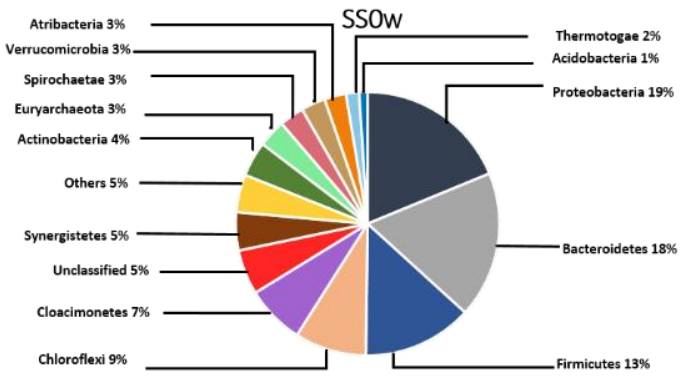
Figure ESM-1. Portuguese military map showing the São Domingos mining area and the sampling sites (see coordinates in Table 1).

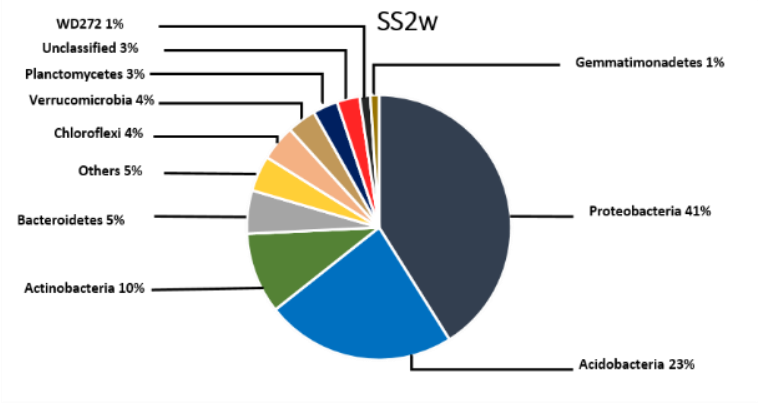
Figure ESM-2. Principal component analysis of water's physicochemical characteristics at sampled sites, using normalized data (adapted from Ettamimi et al. 2019).

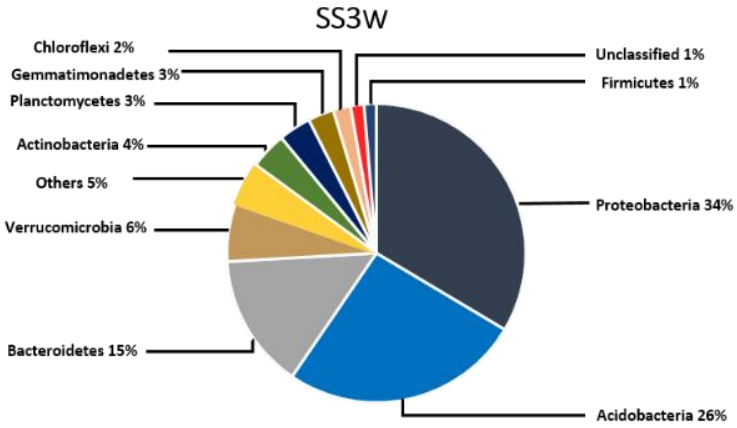
Table ESM-1. Number of sequence reads and operational taxonomic units OTUs retrieved from each sediment sample.

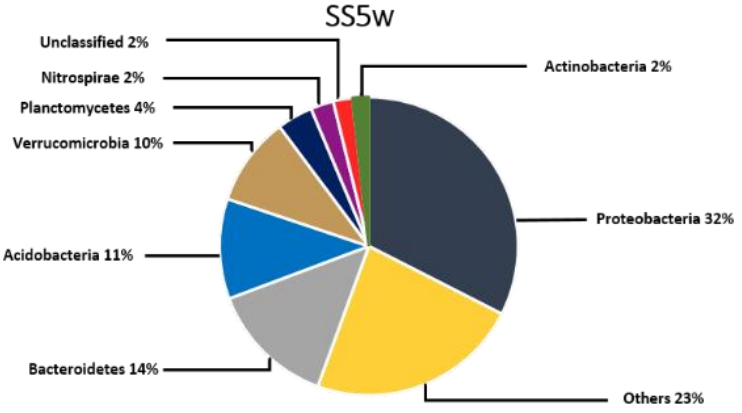
Table ESM-2. Relative abundances (%) of the 30 most abundant prokaryotes among all the sediment samples collected at São Domingos mine during winter. The names are shown based on the phylum and the genus assignment or the phylum and the lowest possible taxonomic assignment (class (c__), order (o__) or family (f__)) together with the OTU number.

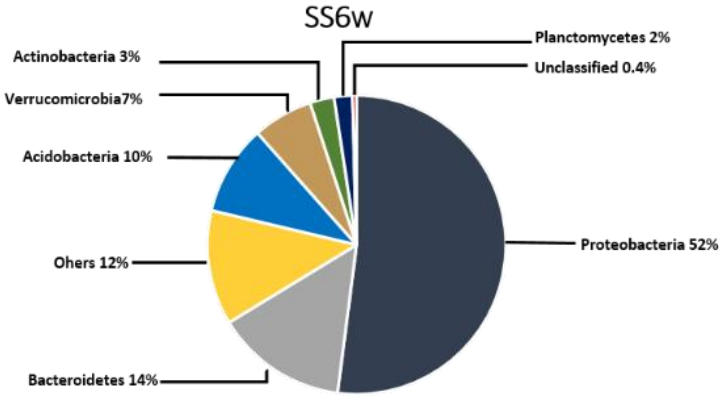
Table ESM-3. Relative abundances (%) of the 30 most abundant prokaryotes among all the sediment samples collected at São Domingos mine during summer. The names are shown based on the phylum and the genus assignment or the phylum and the lowest possible taxonomic assignment (class (c__), order (o__) or family (f__)) together with the OTU number.


SS0w
Atribacteria 3%
Verrucomicrobia 3%
Spirochaetae 3%
Euryarchaeota 3%
Actinobacteria 4%
Others 5%
Synergistetes 5%
Unclassified 5%
Cloacimonetes 7%
Chloroflexi 9%
Thermotogae 2%
Acidobacteria 1%
Proteobacteria 19%
Bacteroidetes 18%
Firmicutes 13%


SS2w
WD272 1%
Unclassified 3%
Planctomycetes 3%
Verrucomicrobia 4%
Chloroflexi 4%
Others 5%
Bacteroidetes 5%
Actinobacteria 10%
Gemmatimonadetes 1%
Proteobacteria 41%
Acidobacteria 23%


SS3W
Chloroflexi 2%
Gemmatimonadetes 3%
Planctomycetes 3%
Actinobacteria 4%
Others 5%
Verrucomicrobia 6%
Bacteroidetes 15%
Unclassified 1%
Firmicutes 1%
Proteobacteria 34%
Acidobacteria 26%


SS5w
Unclassified 2%
Nitrospirae 2%
Planctomycetes 4%
Verrucomicrobia 10%
Acidobacteria 11%
Bacteroidetes 14%
Actinobacteria 2%
Proteobacteria 32%
Others 23%


SS6w
Actinobacteria 3%
Verrucomicrobia7%
Acidobacteria 10%
Ohers 12%
Bacteroidetes 14%
Planctomycetes 2%
Unclassified 0.4%
Proteobacteria 52%

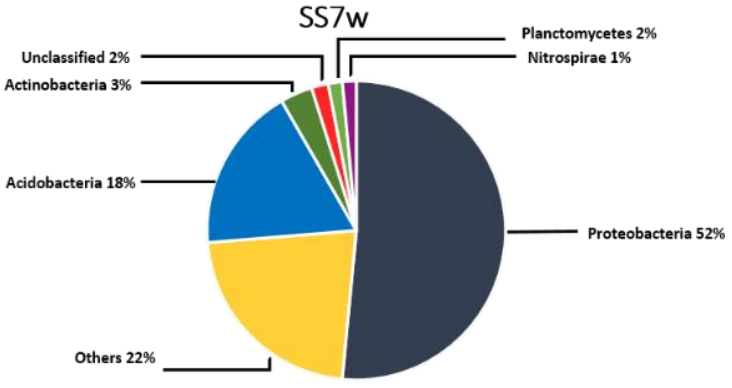

SS7w
Unclassified 2%
Actinobacteria 3%
Acidobacteria 18%
Others 22%
Planctomycetes 2%
Nitrospirae 1%
Proteobacteria 52%

Fig. 1a

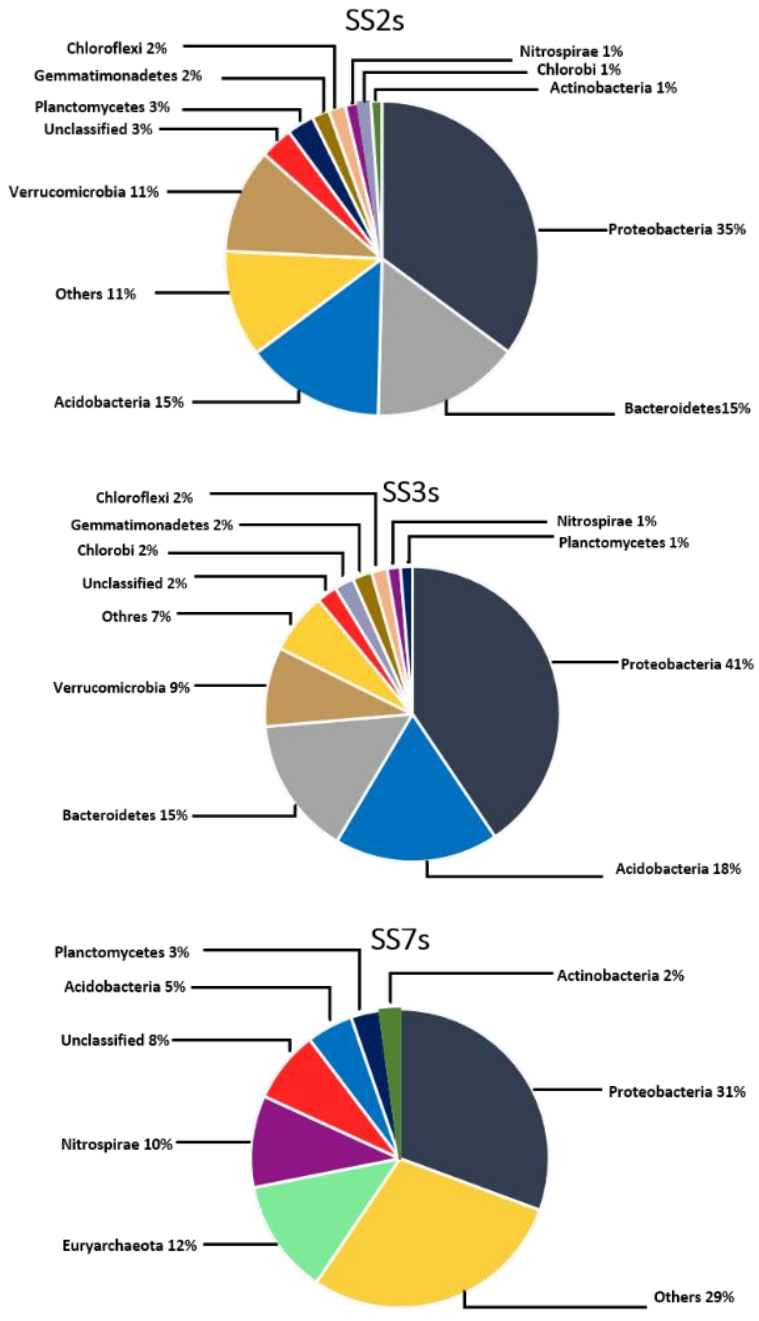
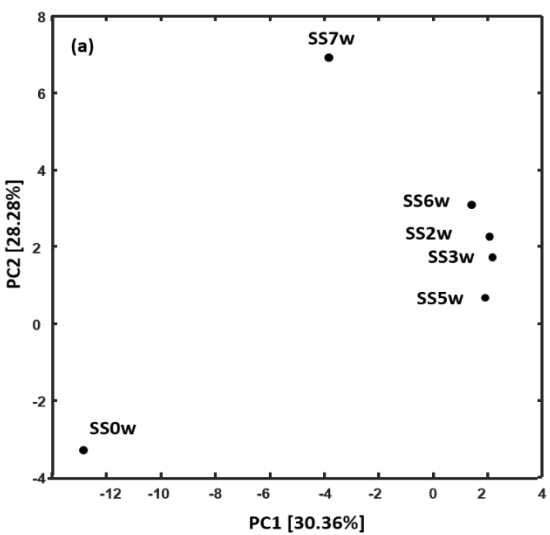

SS2s
Chloroflexi 2%
Gemmatimonadetes 2%
Planctomycetes 3%
Unclassified 3%
Verrucomicrobia 11%
Others 11%
Acidobacteria 15%
Nitrospirae 1%
Chlorobi 1%
Actinobacteria 1%
Proteobacteria 35%
Bacteroidetes15%
SS3s
Chloroflexi 2%
Gemmatimonadetes 2%
Chlorobi 2%
Unclassified 2%
Othres 7%
Verrucomicrobia 9%
Bacteroidetes 15%
Nitrospirae 1%
Planctomycetes 1%
Proteobacteria 41%
Acidobacteria 18%
SS7s
Planctomycetes 3%
Acidobacteria 5%
Unclassified 8%
Nitrospirae 10%
Euryarchaeota 12%
Actinobacteria 2%
Proteobacteria 31%
Others 29%

Fig. 1b


(a)
SS7w
SS6w
SS2w
SS3w
SS5w
SS0w
PC2 [28.28%]
PC1 [30.36%]
8
6
4
2
0
-2
-4
-12
-10
-8
-6
-4
-2
0
2
4

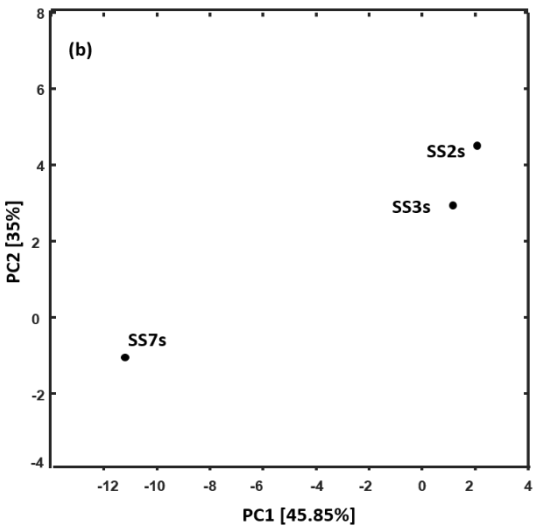

(b)
SS2s
SS3s
SS7s
PC2 [35%]
PC1 [45.85%]
8
6
4
2
0
-2
-4
-12
-10
-8
-6
-4
-2
0
2
4

Fig. 2

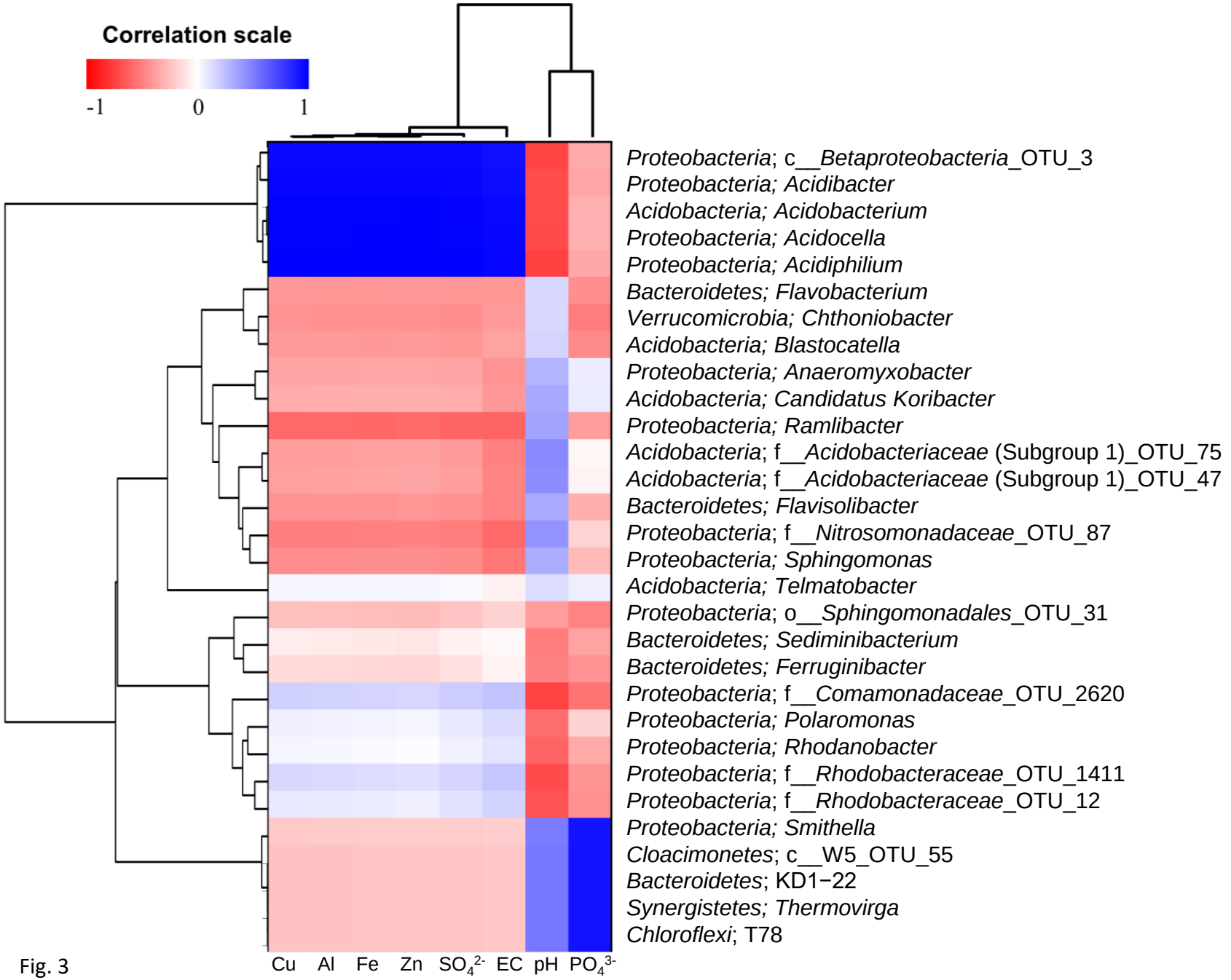

Correlation scale
-1
0
1
Proteobacteria; c__Betaproteobacteria_OTU_3
Proteobacteria; Acidibacter
Acidobacteria; Acidobacterium
Proteobacteria; Acidocella
Proteobacteria; Acidiphilium
Bacteroidetes; Flavobacterium
Verrucomicrobia; Chthoniobacter
Acidobacteria; Blastocatella
Proteobacteria; Anaeromyxobacter
Acidobacteria; Candidatus Koribacter
Proteobacteria; Ramlibacter
Acidobacteria; f__Acidobacteriaceae (Subgroup 1)_OTU_75
Acidobacteria; f__Acidobacteriaceae (Subgroup 1)_OTU_47
Bacteroidetes; Flavisolibacter
Proteobacteria; f__Nitrosomonadaceae_OTU_87
Proteobacteria; Sphingomonas
Acidobacteria; Telmatobacter
Proteobacteria; o__Sphingomonadales_OTU_31
Bacteroidetes; Sediminibacterium
Bacteroidetes; Ferruginibacter
Proteobacteria; f__Comamonadaceae_OTU_2620
Proteobacteria; Polaromonas
Proteobacteria; Rhodanobacter
Proteobacteria; f__Rhodobacteraceae_OTU_1411
Proteobacteria; f__Rhodobacteraceae_OTU_12
Proteobacteria; Smithella
Cloacimonetes; c__W5_OTU_55
Bacteroidetes; KD1−22
Synergistetes; Thermovirga
Chloroflexi; T78
Cu
Al
Fe
Zn
SO_4^{2-}
EC
pH
PO_4^{3-}

Fig. 3

Table 1.

Site ID	Sediment sample				
	winter	**summer**	**Site name**	**Latitude/ longitude**	**Brief description of the site**
S0	**SS0w**	-	*Mosteirão stream*	37.635849, -7.516451	Mosteirão stream - a small brook (dry in the summer). Not impacted by AMD until it merges with the São Domingos stream, thus used as reference.
S2	**SS2w**	**SS2s**	*Chança reservoir*	37.612376, -7.494921	Large reservoir with dammed water from Chanca´s river into which the contaminated water flows. Low AMD contamination.
S3	**SS3w**	**SS3s**	*Upstream Chança reservoir*	37.624352, -7.514251	Transition zone between the contaminated stream and Chança´s reservoir. Low to moderate AMD contamination.
S5	**SS5w**	-	*Telheiro*	37.636529, -7.513189	Site where the water coming from Site 0 is mixed with the water coming from Site 6 (dry in the summer). A mixture of uncontaminated and AMD contaminated water.
S6	**SS6w**	-	*São Domingos stream*	37.635274, -7.513640	A small stream downstream site 7, after merging with several non-contaminated water streams (dry in the summer). Moderate to high AMD contamination.
S7	**SS7w**	**SS7s**	*Tapadinha*	37.659461, -7.505471	Medium-sized dammed reservoir containing reddish brown acidic water. Very high AMD contamination.

Table 2.

	Site ID	T (C°)	pH	EC (µs/cm)	DO (mg/L)	SO_4^{2-} (mg/l)	PO_4^{3-} (mg/l)	Fe (mg/l)	Zn (mg/l)	Cu (mg/l)	Al (mg/l)
Winter	**S0**	16.6	7.59	369	8.04	61	12.64	<LOD	<LOD	<LOD	<LOD
	S2	14.1	6.65	173	8.73	36	4.85	<LOD	<LOD	<LOD	0.32±0.03
	S3	14.5	6.40	205	9.35	35	1.22	<LOD	0.26±0.01	0.121±0.002	0.34±0.01
	S5	16.8	5.63	557	8.55	122	0.17	3.2±0.4	0.78±0.01	0.781±0.005	6.6±0.1
	S6	17.1	3.06	795	8.39	514	0.28	14.6±0.7	2.98±0.03	2.80±0.03	22.9±0.5
	S7	17.7	2.25	2883	9.00	3477	0.10	144 ±1	31.8±0.2	23.15±0.04	202±2
Summer	**S0**	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry
	S2	26.9	7.65	267	7.50	34	0.07	0.265±0.001	<LOD	<LOD	<LOD
	S3	28.2	7.50	267	8.10	38	0.04	0.299±0.003	<LOD	<LOD	<LOD
	S5	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry
	S6	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry
	S7	32.1	2.26	6750	7.48	6256	0.61	770±4	194.2±0.8	81±1	810±8

*Limits of detection (LOD) for metals analysis: Copper = 0.034 mg/L; Iron = 0.211 mg/L; Zinc = 0.122 mg/L; Aluminum = 0.098 mg/L

Table 3. Shannon and Simpson diversity indexes calculated for all sediments samples with (**a**) non-normalized OTUs tables and (**b**) normalized OTUs tables, using the R package RAM - R for Amplicon-Sequencing-Based Microbial-Ecology (Chen et al, 2018). The pH of samples is also shown (in the first row). Colour scales were obtained by conditional formatting each row independently using the Excel software (Microsoft Office).

	Winter samples						Summer samples		
	SS0w	**SS2w**	**SS3w**	**SS5w**	**SS6w**	**SS7w**	**SS2s**	**SS3s**	**SS7s**
pH	7.59	6.65	6.40	5.63	3.06	2.25	7.65	7.50	2.26
a) raw OTUs table									
Shannon	4.98770	5.74562	5.90112	6.24430	5.67346	4.41475	6.31123	6.19973	2.75324
Simpson	0.98237	0.99259	0.99299	0.99566	0.98944	0.97693	0.99642	0.99555	0.83890
b) normalized OTUs table									
Shannon	4.98464	5.74509	5.90186	6.24401	5.67245	4.41475	6.31123	6.20008	2.75429
Simpson	0.98233	0.99259	0.99301	0.99565	0.98940	0.97693	0.99642	0.99555	0.83909

Prokaryotic diversity in stream sediments affected by acid mine drainage

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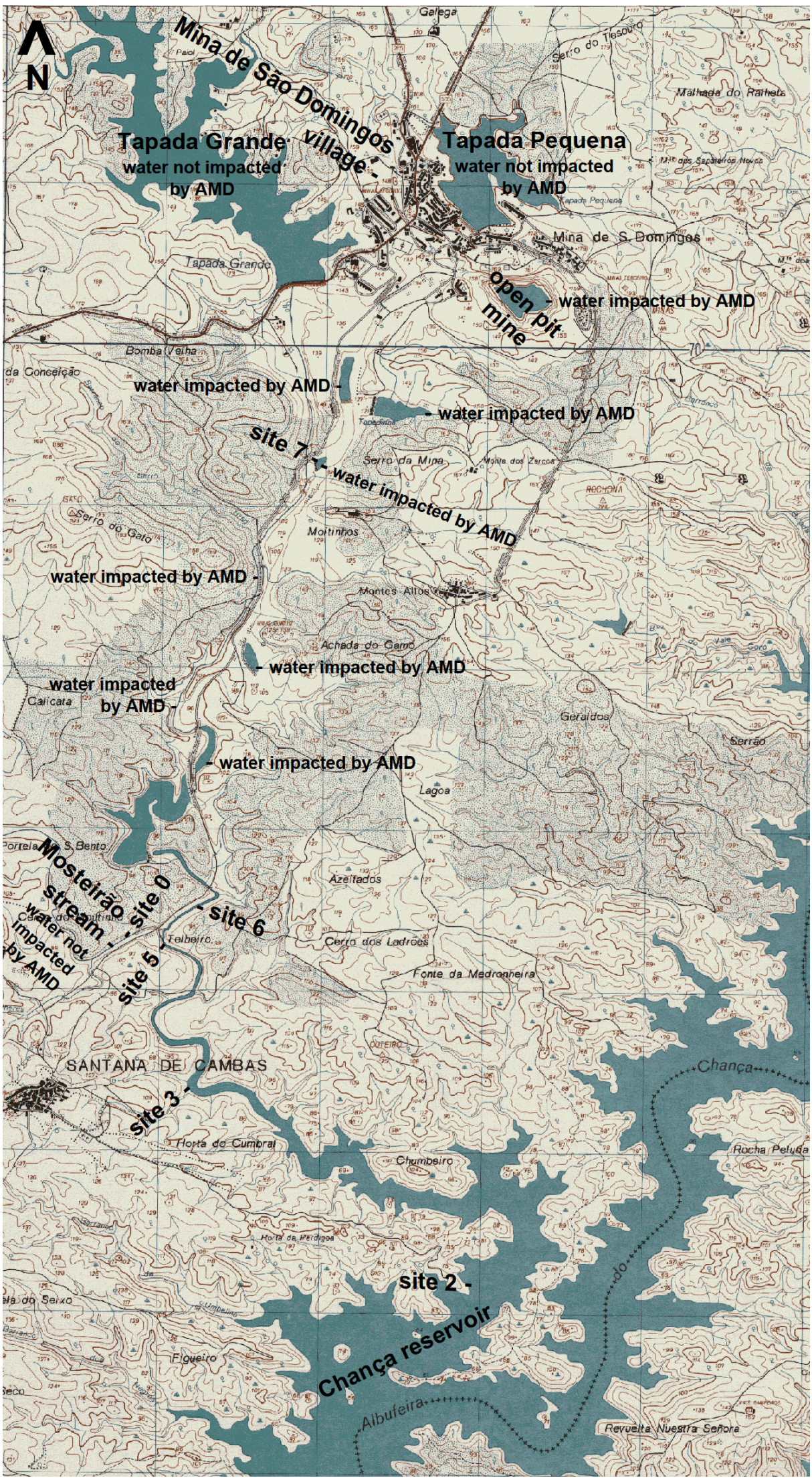

Figure ESM-1. Portuguese military map showing the São Domingos mining area and the sampling sites (see coordinates in Table 1).

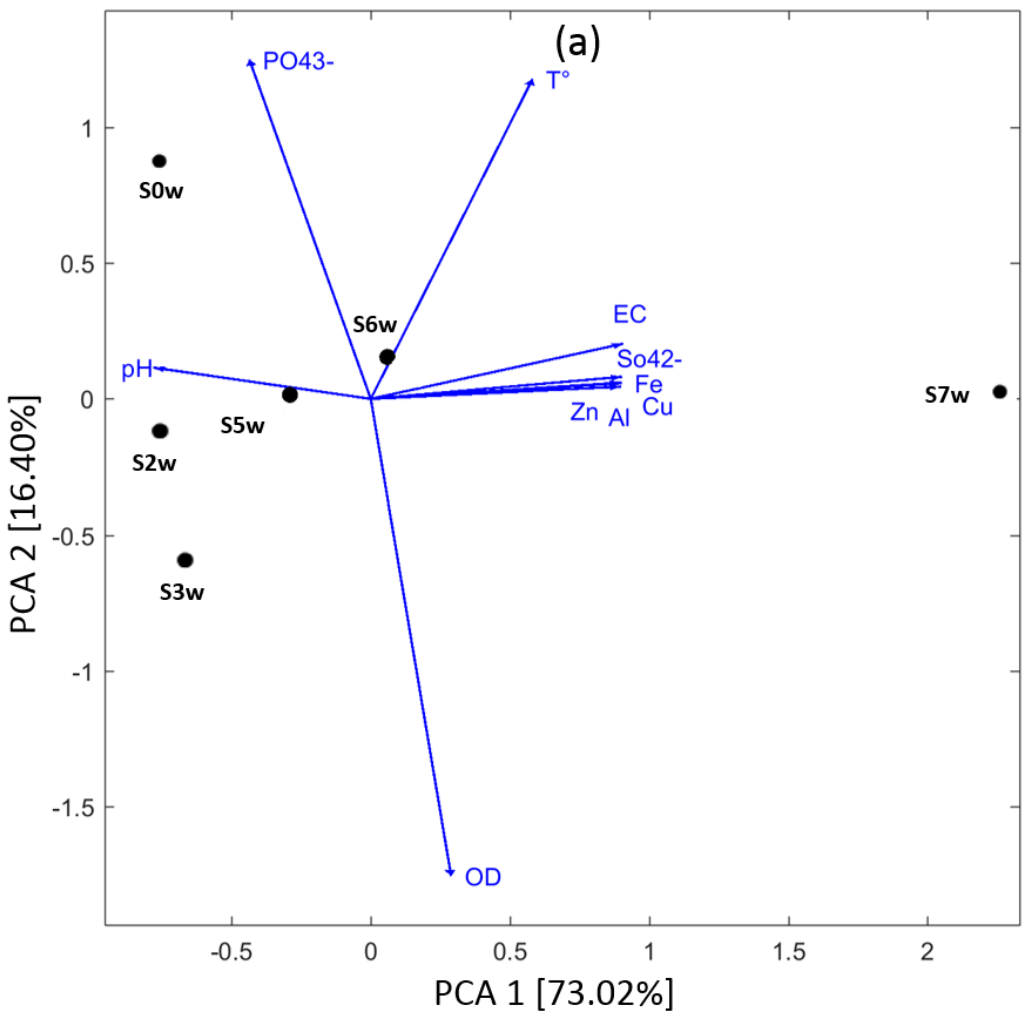

Figure ESM-2. Principal component analysis of water's physicochemical characteristics at sampled sites, using normalized data (adapted from Ettamimi et al. 2019).

Table ESM-1. Number of sequence reads and operational taxonomic units OTUs retrieved from each sediment sample.

	Sediments	Sequencing ID	N.er of reads	N.er of OTUs
Winter	SS0w	16SAMP−16290	32932	273
	SS2w	16SAMP−16293	33591	387
	SS3w	16SAMP−16292	35791	372
	SS5w	16SAMP−16291	31550	404
	SS6w	16SAMP−16294	36057	290
	SS7w	16SAMP−16289	8775	177
Summer	SS0s	Dry	-	-
	SS2s	MG171016−5	19381	1282
	SS3s	MG171016−6	19244	1273
	SS5s	Dry	-	-
	SS6s	Dry	-	-
	SS7s	MG171016−7	26940	186

Table ESM-2. Relative abundances (%) of the 30 most abundant prokaryotes among all the sediment samples collected at São Domingos mine during winter. The names are shown based on the phylum and the genus assignment or the phylum and the lowest possible taxonomic assignment (class (c__), order (o__) or family (f__)) together with the OTU number.

Site name	SS0w	SS2w	SS3w	SS5w	SS6w	SS7w
Proteobacteria; Acidiphilium	0.0	0.2	0.1	0.1	1.3	14.9
Proteobacteria; Acidocella	0.0	0.0	0.0	0.0	0.1	5.8
Acidobacteria; Acidobacterium	0.0	0.1	0.0	0.0	0.1	5.4
Proteobacteria; Acidibacter	0.0	0.7	0.3	0.2	0.3	4.3
Proteobacteria; c__Betaproteobacteria_OTU_3	0.0	0.9	0.0	0.0	0.6	3.8
Proteobacteria; f__Rhodobacteraceae_OTU_12	0.1	0.1	0.2	1.0	5.7	1.3
Proteobacteria; f__Comamonadaceae_OTU_2620	0.1	0.5	0.5	0.4	1.9	0.8
Proteobacteria; f__Rhodobacteraceae_OTU_1411	0.0	0.1	0.1	0.2	2.6	0.7
Acidobacteria; Telmatobacter	0.0	2.5	0.1	0.0	0.0	0.7
Proteobacteria; Rhodanobacter	0.0	0.2	0.0	0.0	3.5	0.5
Proteobacteria; Polaromonas	0.5	0.2	0.2	0.2	1.7	0.5
Bacteroidetes; Ferruginibacter	0.3	0.0	0.6	0.8	2.2	0.3
Proteobacteria; Sphingomonas	0.0	3.2	3.6	1.1	1.3	0.3
Bacteroidetes; Sediminibacterium	0.0	0.7	0.2	0.2	2.0	0.3
Proteobacteria; o__Sphingomonadales_OTU_31	0.0	0.8	0.2	1.0	1.7	0.2
Proteobacteria; Smithella	3.4	0.0	0.0	0.0	0.0	0.1
Bacteroidetes; KD1−22	7.8	0.0	0.0	0.0	0.0	0.0
Cloacimonetes; c__W5_OTU_55	3.6	0.0	0.0	0.0	0.0	0.0
Acidobacteria; Blastocatella	0.2	0.0	3.2	3.8	1.1	0.0
Chloroflexi; T78	3.7	0.0	0.0	0.0	0.0	0.0
Proteobacteria; Ramlibacter	0.1	2.6	2.7	3.2	1.2	0.0
Acidobacteria; f__Acidobacteriaceae (Subgroup 1)_OTU_47	0.0	3.8	4.5	0.0	0.0	0.0
Proteobacteria; f__Nitrosomonadaceae_OTU_87	0.0	2.4	2.1	0.8	0.6	0.0
Bacteroidetes; Flavobacterium	0.1	0.1	2.7	1.4	1.0	0.0
Bacteroidetes; Flavisolibacter	0.0	0.8	2.5	0.8	0.4	0.0
Verrucomicrobia; Chthoniobacter	0.0	0.3	1.6	2.1	0.7	0.0
Synergistetes; Thermovirga	4.2	0.0	0.0	0.0	0.0	0.0
Acidobacteria; Candidatus Koribacter	0.0	3.1	0.6	0.2	0.3	0.0
Acidobacteria; f__Acidobacteriaceae (Subgroup 1)_OTU_75	0.0	2.1	2.0	0.1	0.0	0.0
Proteobacteria; Anaeromyxobacter	0.1	2.6	0.4	0.3	0.6	0.0

Table ESM-3. Relative abundances (%) of the 30 most abundant prokaryotes among all the sediment samples collected at São Domingos mine during summer. The names are shown based on the phylum and the genus assignment or the phylum and the lowest possible taxonomic assignment (class (c__), order (o__) or family (f__)) together with the OTU number.

Site name	SS2s	SS3s	SS7s
Proteobacteria; f__RCP1−48_OTU_6	0.0	0.0	36.4
Nitrospirae; Leptospirillum	0.0	0.0	13.6
k__Unclassified_OTU_12; k__Unclassified_OTU_12	0.0	0.0	9.9
Euryarchaeota; Thermoplasma	0.0	0.0	9.1
Acidobacteria; f__Acidobacteriaceae (Subgroup 1)_OTU_45	0.0	0.0	4.6
Euryarchaeota; f__Ferroplasmaceae_OTU_36	0.0	0.0	4.6
Planctomycetes; o__CPla−3 termite group_OTU_38	0.0	0.0	1.8
Proteobacteria; Acidibacter	0.0	0.0	1.4
Acidobacteria; Bryobacter	2.3	1.8	0.0
Acidobacteria; Blastocatella	2.7	1.3	0.0
Bacteroidetes; f__Cytophagaceae_OTU_51	1.1	2.8	0.0
Bacteroidetes; f__Cytophagaceae_OTU_219	1.8	1.8	0.0
Proteobacteria; f__Comamonadaceae_OTU_142	2.0	0.7	0.0
Proteobacteria; Anaeromyxobacter	0.6	1.6	0.0
Proteobacteria; f__Comamonadaceae_OTU_468	0.9	1.4	0.0
Verrucomicrobia; Opitutus	1.2	1.0	0.0
Proteobacteria; Sulfuritalea	1.0	1.2	0.0
Proteobacteria; f__M20−Pitesti_OTU_65	0.5	1.3	0.0
Acidobacteria; Geothrix	0.0	1.7	0.0
Acidobacteria; f__Acidobacteriaceae (Subgroup 1)_OTU_91	0.4	1.2	0.0
Proteobacteria; c__Betaproteobacteria_OTU_86	0.3	1.2	0.0
Bacteroidetes; f__WCHB1−53_OTU_74	0.3	1.1	0.0
Proteobacteria; f__Rhodocyclaceae_OTU_97	1.2	0.3	0.0
Proteobacteria; c__Betaproteobacteria_OTU_88	0.9	0.6	0.0
Bacteroidetes; f__Cytophagaceae_OTU_176	1.3	0.1	0.0
Verrucomicrobia; c__S−BQ2−57 soil group_OTU_113	0.9	0.5	0.0
Proteobacteria; f__Nitrosomonadaceae_OTU_130	0.5	0.8	0.0
Proteobacteria; f__Nitrosomonadaceae_OTU_2413	0.4	0.9	0.0
Proteobacteria; A0837	0.6	0.7	0.0
Acidobacteria; Candidatus Koribacter	0.3	0.7	0.0