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Abstract

Mining activities frequently leave a legacy of residues that remain in the area for long periods causing the pollution of surroundings. We studied on a 100 year-old mine, the behaviour of potentially toxic elements (PTEs) and their ecotoxicological impact on activity and diversity of microorganisms. The PTEs contamination assessment allowed the classification of the materials as highly (reference- and contaminated-samples) and very highly polluted (illegal spill of olive mill wastes (OMW), tailings and dumps). OMW presented the lowest enzymatic activities while tailings and dumps had low dehydrogenase and arylsulfatase activities. All the alpha diversity indices studied were negatively impacted in dumps. Tailings had lower Chao1 and PD whole tree values as compared to those of reference-samples. Beta diversity analysis showed similar bacterial community composition for reference- and contaminated-samples, significantly differing from that of tailings and dumps. The relative abundance of Gemmatimonadetes, Bacteroidetes and Verrucomicrobia was lower in OMW, tailings and dumps as compared to reference-samples. Fifty-seven OTUs were selected as responsible of the changes observed between samples. This study highlights that assessing the relationship between physicochemical properties and microbial diversity and activity gives clues about ongoing regulating processes that can be helpful for stakeholders to define an appropriate management strategy.

Keywords: Potentially toxic elements, mining soil, enzyme activities, high-throughput sequencing, microbial ecotoxicology,

1. Introduction

Mining activities are found all over the world because they provide access to mineral resources that fuel various industrial activities in both developed and in developing countries. Although recognized of great importance for the world gross domestic product, mining operations are often viewed as an important source of pollution with negative impact on the environment. During the processes of mineral extraction and preparation, large amounts of ore wastes and debris are commonly accumulated in the proximity of the mining operation site. These materials are essentially fractured rocks and soil devoid of vegetation, characterized by high concentrations of heavy metals and metalloids. Consequently, the environment is drastically transformed in highly polluted barren areas (Martín Duque et al., 2015; Sánchez-Donoso et al., 2019), which can be toxic to human health and other life, including plants and microorganisms (Giller et al., 1998; Nagajyoti et al., 2010; Tchounwou et al., 2012). Furthermore, potentially toxic elements (PTEs) from these polluted areas can transfer to surrounding aquatic and terrestrial compartments via leaching or runoff (Elmayel et al., 2020; Fernández-Martínez et al., 2019; Jung and Thornton, 1996; Kisková et al., 2018), disperse in the atmosphere (Esbrí et al., 2020; Naharro et al., 2020, 2018), and indirectly contribute to pollutant dissemination. The recent interest in the reclamation of abandoned mining sites in arid and semiarid regions for agricultural purposes highlights the need to understand the biogeochemical processes contributing to soil health and fertility (Favas et al., 2018; Higueras et al., 2019b; Krzaklewski and Pietrzykowski, 2002; Mendez et al., 2008)

Microorganisms such as bacteria and fungi are key players in soil ecosystem services. They are involved in multiple geochemical cycles, influence plant growth and contribute to climate regulation and soil restoration, among others (Ayangbenro et al., 2018; Cavicchioli et al., 2019; Domeignoz-Horta et al., 2018, 2015; Van Der Heijden et al., 2008). Toxicity from heavy metals and metalloids can compromise their growth and survival, with enormous

consequences on ecosystem functioning (Bell et al., 2005; Delgado-Baquerizo et al., 2020, 2017, 2016b, 2016a; Domeignoz-Horta et al., 2020; Griffiths and Philippot, 2013; Philippot et al., 2013; Wagg et al., 2014). However, some microorganisms have developed several resistance mechanisms to cope with their toxic effects such as extra and intracellular sequestration, exclusion by permeability barriers, enzymatic detoxification, efflux-pumps and specific reduction of cellular targets' sensibility (Hobman et al., 2007; Nies, 2003; Rouch et al., 1995). In this frame, soil microorganisms constitute useful bio indicators of soil quality (Alvarenga et al., 2018; Thiele-Bruhn et al., 2020). Measurement of microbial activity through analysis of soil extracellular enzymatic activities has long been used as indicator of soil disturbance and can allow the assessment of the impact of environmental contaminates on soil microbial processes (Campos et al., 2018a, 2018b; Chu et al., 2007; Elmayel et al., 2020; Higueras et al., 2019a; Hinojosa et al., 2004). Additionally, the implementation of state of the art high throughput sequencing approaches could provide a high-resolution analysis of the ecotoxicological effects of PTEs on the composition and diversity of soil microbial populations (Fernandes et al., 2018; Gallego et al., 2019; Jacquiod et al., 2018; Kisková et al., 2018).

The province of Ciudad Real, in the center of the Iberian Peninsula, is scattered with abandoned and decommissioned mines (Bravo et al., 2019; García-Lorenzo et al., 2019; Higueras et al., 2017, 2012; Martín-Crespo et al., 2015; Rodríguez et al., 2009; Ruiz et al., 2009). In this regard, the San Quintín mine site (Cabezarados, Ciudad Real, Spain) offers an unprecedented opportunity to shed light on this topic. This area covers about 100 Ha, with a complex long-lasting history of mining and mineralurgical operations (Fig. 1). Between the years 1888 and 1923, three mine shafts and a rudimentary froth flotation plant operated to obtain Ag-rich galena (PbS) concentrates, leaving a first legacy of dumps, very heterogeneous in grain size and containing high concentrations of Pb, and Zn (García-Lorenzo et al., 2019).

In 1973, a second froth flotation plant aimed to recuperate the sphalerite (ZnS) as well as residual Ag-rich galena was established. During this period, the froth flotation process favored the conversion of the dumps in wastes dams, which accumulated the residues from this reprocessing. Additionally, cinnabar (HgS) from the adjacent Hg mines of Almadén (Higueras et al., 2006; Tejero et al., 2015) was accumulated in the area during the last years of activity of the froth flotation plant (1988-1990). In 2000, olive-oil milling wastes (OMW) were illegally applied over the soils without any remediation interest. Currently, soil and waste materials from the area are used in local road pavements, resulting in a redistribution of the soil. Consequently, the area is deeply polluted with Pb, Zn and associated elements (García-Lorenzo et al., 2019; Higueras et al., 2017; Martín-Crespo et al., 2015; Rodríguez et al., 2009; Ruiz et al., 2009).

The characterization of the behavior of PTEs from heavily degraded mining soils and their impact on the environment is the first step for planning restoration strategies in contaminated areas. We hypothesized that decades of exposure to different levels of pollutants have exerted a selective pressure on microbial communities in the San Quintín mine area. Within this context, this study aims to investigate the link between geochemical characteristics and microbial communities in a long-term polluted mining site and assess the effects of PTEs on the activity, composition and diversity of microbial communities through enzymatic analysis and high throughput sequencing.

2. Materials and Methods

2.1 Samples collection

Twenty samples of circa 800 g corresponding to the first 10-15 cm and relative to five materials were collected (four independent replicates per material, each replicate was a

114 composite made out of three subsamples collected and homogenized *in situ*). The five
115 different materials included: non-directly polluted soil taken from the surrounding area and
116 used as local background (reference-samples); PTEs polluted soil from mine operations (via
117 leaching and runoff) and collected within the precincts of the mining site (contaminated-
118 samples); olive-oil mill wastes residues spilled in the area (OMW); processed waste
119 accumulated in the dams (tailings); and mine wastes without any treatment (dumps). Samples
120 were immediately transported to the laboratory and sieved (<2 mm) within 24 hours. Samples
121 were divided in three aliquots and stored at -4ºC (physicochemical and enzyme analysis)
122 (Peoples and Koide, 2012) or -20ºC (DNA extraction) until use. Location and main
123 characteristics of sampling points are described in Fig.1 and Table 1.

124

125 **2.2 Physicochemical analysis**

126 Physicochemical analyses were conducted in the Laboratory of Soil Biogeochemistry at
127 the EIMIA-UCLM, Almadén (Spain). Electric conductivity and pH were determined using
128 ISO-10390 (ISO-10390, 2005) and ISO-7888 (ISO-11265, 1994) protocols, respectively.
129 Analyses were performed on 2 g dwt of sieved samples. Texture was determined on dried
130 samples treated with a solution of 3% (v/v) H_2O_2 for 48 h to remove organic matter. Samples
131 were then wet and sieved at 2 mm and analyzed with a Fritsch Analysette MicroTec Plus 22
132 (Fritsch, Germany). A textural classification triangle plot was used to identify the
133 granulometry of samples (Gerakis and Baer, 1999). Organic matter (OM) was determined
134 using the Walkley-Black method (Walkley and Black, 1934).

135 Elemental concentration analyses were performed from ground dried samples with
136 particle length minor to 100 µm determined by means of X-Ray Fluorescence spectroscopy,

using an Epsilon 1 device (Malvern Panalytical, England). Pollution Load Index (PLI) (Jorfi et al., 2017) was calculated according to the equation:

$$PI = Cn/Bn$$

$$PLI = \sqrt{PI1\tilde{n}PI2 \dots PIn}$$

Where PI is the single factor pollution index of each metal, C_n and B_n is the concentration of metal in the sample and background, respectively (mg Kg^{-1}). Reference average values from this study were used as local background level. The values of PLI allow the qualification of samples as follows: PLI < 2: moderately to unpolluted; 2 ≤ PLI < 4: moderately polluted; 4 ≤ PLI < 6: highly polluted; PLI > 6: very highly polluted.

2.3 Enzyme analysis

Enzyme activities were measured through colorimetric techniques using a Biochrom Libra S60 spectrophotometer (Biochrom, United Kingdom). Dehydrogenase (DHA) activity was measured according to the method described by Casida (1977), with slight modifications. Acid and alkaline phosphomonoesterase (PhA 6.5 and PhA 11, respectively) activities were determined following Tabatabai and Bremner (1969) method. Arylsulfatase (ARS) activity was measured as described by Tabatabai and Bremmer, (1970) and β-galactosidase (β-Gal) activity was assayed according to Eivazi and Tabatabai (1988).

Functional diversity from selected enzymes was assessed with the geometric mean index (GMean) (Lessard et al., 2014) calculated as follow:

$$GMean = \left(\prod_{i=1}^{n} y_i \right)^{\frac{1}{n}}$$

Where y_i is the mean value for each enzyme activity, n is the total number of enzymes.

160 **2.4 Microbiological analysis**

161 DNA was extracted using the Power soil DNA isolation kit (Qiagen). The extracted DNA
162 was quantified by using fluorescent dye of a Quant-iT™ PicoGreen® dsDNA assay kit
163 (Invitrogen). The diversity of the bacterial community was determined by high throughput
164 sequencing of 16S rRNA amplicons generated in a two-step PCR. First PCR reaction was
165 performed using the universal bacterial primers U341_F-805_R with overhang adapters
166 (forward adapter: TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG, reverse adapter:
167 GTCTCGTGGGCTCGGAGATGTGTATA AGAGACAG). The reaction mixture contained 2
168 ng of DNA as template, 7.5 µL of 2X Phusion High Fidelity PCR Mastermix (Thermo
169 Scientific, Waltham, MA, USA), 250 ng of T4 gene 32 protein (MP Biomedicals, Santa Ana,
170 CA, USA), 0.375 µL of each primer (10 µM) and ultrapure sterile water to a total volume of
171 15 µL. Thermal conditions were 3 min at 98°C, 25 cycles at 98° C for 30 sec, 55° C for 30 sec
172 and 72° C for 30 sec followed by a final extension of 10 min at 72ºC. Duplicates of each
173 amplicon were pooled and then 6 µL aliquot was used as template in a second PCR carried
174 out with multiplexed primers containing the universal overhang adaptors and specific
175 barcodes and using a 384 Nextera XT index kit (Illumina, San Diego, CA, USA). The reaction
176 mixture was carried out in 30 µL reaction volumes containing 2.5 µL sterile water, 15 µL 2X
177 Phusion HF master mix (Thermo Scientific, Waltham, MA, USA), 250 ng of T4 gene 32
178 protein (MP Biomedicals, Santa Ana, CA, USA), 3 µL of each primer (10 µM) and 6 µL of
179 the step-one PCR product. The thermal cycling was 98°C for 3 min, followed by eight cycles
180 of 98° C for 30 sec, 55ºC for 30 sec and 72° C for 30 sec, with a final extension of 72º C for
181 10 min. The size of the amplicons was verified by electrophoresis on a 2 % agarose gel.
182 Amplicons were purified (amplicon library purification, PicoGreen® quantification and
183 pooling) and sequenced (Illumina MiSeq 2 x300bp) by Microsynth (Balgach, Switzerland).
184 Amplicons were normalized (SequalPrep™ kit), purified (Pippin prep) and sequenced by

185 Microsynth (Switzerland). The sequence data was analyzed using an in-house developed
186 Phyton notebook pipeline together with different bioinformatics tools: 16S rDNA sequences
187 were assembled using the PEAR software (Zhang et al., 2014) with the default settings;
188 further quality checks were conducted using the QIIME 1 pipeline (Caporaso et al., 2010a);
189 sequences shorter than 350 bp were removed; reference-based and *de novo* chimera detection,
190 as well as clustering in OTUs were performed with the VSEARCH software (Rognes et al.,
191 2016) using appropriate reference databases (Greengenes' representative set of sequences for
192 16S rDNA) with a threshold placed at 94 % identity; representative sequences for each OTU
193 were aligned using PyNAST (Caporaso et al., 2010b); phylogenetic trees were constructed
194 using FastTree (Price et al., 2009); taxonomy was assigned using UCLUST (Edgar, 2010) and
195 the latest released Greengenes database (v.05/2013, McDonald et al., 2012) for 16S rDNA
196 sequences; sequences were deposited in the GenBank to the sequence read archive (SRA)
197 under the accession number PRJNA646888. A range of bacterial α-diversity indices
198 pertaining to richness (Chao1, observed species), evenness (Simpson reciprocal, equitability,
199 dominance, Shannon) and relatedness (PD whole tree) were calculated based on rarefied
200 tables (13,000 sequences per sample). Bray Curtis distance matrices were also computed to
201 detect changes in the composition of microbial communities. Canonical Analysis of Principal
202 Coordinates (CAP) of OTU Bray Curtis distance matrices were also performed and plotted.
203 The relative abundance of the different bacterial phyla was also determined using a
204 comparative bar chart.

205

206 **2.5 Statistical analysis**

207 All statistical analyses were performed in R (http://www.r-project.org). The normality of
208 the data and residuals was checked (Shapiro Wilk's with $p>0.05$) and the homogeneity of
209 variances was verified (Levene's test with $p>0.05$). Root square, inverse and log-

transformations on the data were performed when necessary. For parametric distributions, ANOVA followed by Tukey's test and t-student were used to determine differences. For non-parametric distributions, data was compared using Kruskal Wallis test. To detect significant differences in communities' structure among sample types, Permutational multivariate analysis of variance (PermANOVA) was used on Bray Curtis dissimilarity matrix using Adonis function from R package "vegan" (Oksanen et al., 2018).

Integration and visualization of amplicon sequencing and physicochemical and enzymatic analysis data sets were performed through the R package mixOmics (Rohart et al., 2017) using DIABLO (Data Integration Analysis for Biomarker discovery using a Latent component method for Omics studies) in order to identify correlated key omics variables in both datasets (Singh et al., 2019).

3. Results

3.1 Physicochemical and geochemical characterization of samples

Table 2 shows the physicochemical parameters of the samples of the five selected materials. According to their granulometry, samples were classified with some minor variability in sandy loam (reference-, contaminated-samples and dumps), silt loam (tailings) and loamy sand (OMW) (Table 2, Table S1 and Fig. S1). Reference-, contaminated-samples and tailings presented a slightly acidic character while OMW and dumps had lower pH values. EC varied from 62.4 ± 27.8 (reference-samples) to 2282.3 ± 1786.4 mV (dumps). Overall, no significant differences were found in the pH and EC ($p>0.055$), except for the dumps, which presented significant lower pH ($p=0.00371$) and significant higher EC values ($p=0.004$) as compared to the reference-samples. OM ranged from 1.57% ± 0.54 (dumps) to 60.60 % ± 19.67 (OMW). Similar organic content was found in reference-samples, tailings

234 and dumps, while higher values were found in contaminated-samples, although not significant
235 ($p>0.5006$), probably due to their high heterogeneity. On the contrary, significant higher
236 organic matter was found in OMW as compared to the rest of the samples ($p=0.0000271$).

237 The concentration of PTEs for the different samples is shown in Table 3. Among the
238 different PTEs studied, those which were part of the ore (Pb, Zn and Hg) were found at very
239 high concentrations in all samples except reference-samples, with significantly higher
240 concentrations for Pb ($p<0.0043$) and Zn ($p<0.00577$) in dumps and OMW as compared to
241 reference-samples. Similarly, Sb, Co and Cu concentrations were significantly higher in
242 dumps and OMW than in the three other samples ($p<0.0407$). Regarding Mn, Ni and Th,
243 similar concentrations were recorded in all samples and no significant differences were
244 observed ($p>0.05$). A high variability was found for all the PTEs studied. The pollution index
245 for each PTE was calculated (Table S2) and yielded values higher than 100 for Hg (in all
246 samples except reference-samples), Pb (dumps and OMW) and Zn (OMW). Pollution load
247 index (PLI) allowed to classify the samples as follows: reference- and contaminated-samples
248 as highly polluted ($4 \leq PLI < 6$) and OMW, dumps and tailings (from most polluted to less
249 polluted respectively) as very highly polluted ($PLI > 6$) (Table 3).

250

251 **3.2 Enzymatic analysis**

252 Five enzymatic activities including the dehydrogenase (DHA), arylsulfatase (ARS), the
253 acid and alkaline phosphomonoesterase (PhA 6.5 and PhA 11, respectively) and β-
254 galactosidase (β-Gal) were measured in the samples collected from the five sites studied in
255 mining area (Fig. 2 and Table S3). Reference- and contaminated-samples showed similar
256 enzyme activities ($p>0.05$). The lowest enzymatic activities (ARS, PhA 6.5, PhA 11 and β-
257 Gal) were recorded in the OMW, which were significantly different from the values recorded

in reference- and contaminated-samples ($p<0.0398351$) Tailings and dumps had significantly lower DHA value (reduction of about 96 %) and ARS activities than reference-samples ($p<0.0018687$). Tailings had PhA 6.5 activities significantly lower than that of the reference-samples ($p=0.0223$). The geometric mean index was significantly lower in OMW samples ($p=0.0000002$) than that of all the other samples, which were similar ($p>0.06922$) (Fig. S2).

3.3 Bacterial composition and diversity

The composition and diversity of the bacterial community was assessed by high throughput sequencing of 16S rRNA amplicons amplified from DNA extracts. In total, after de-multiplexing and removal of low-quality raw sequence reads, high throughput sequencing generated 1,796,830 high quality sequences with an average sequence length of 461 ± 11bp. Using a threshold at 94% nucleotide sequence identity, these sequences were grouped in 16,299 different OTUs.

A range of α-diversity indices pertaining richness (Chao1), relatedness (PD whole tree) and evenness (Simpson reciprocal) were calculated using rarefied data (Fig. 3). Overall, Chao1, PD whole tree and Simpson reciprocal α-diversity indices were higher in reference-samples than in contaminated-samples, OMW, tailings and dumps, ranked from the highest to the lowest values. Chao1 values were significantly lower in OMW, tailings and dumps ($p<0.027$) while significant differences were found for tailings and dumps in PD whole tree values as compared to reference-samples values ($p<0.019$). Regarding Simpson reciprocal, no significant differences were found between reference-, contaminated-samples, OMW and tailings, and only significant lower values were found in dumps ($p=0.024$).

Differences in the composition of bacterial communities measured in the different samples were assessed by multivariate beta-diversity analysis using Canonical Analysis of Principal

Coordinates (CAP) based on Bray Curtis distances matrices (Fig. 4 & Fig. S3). A good reproducibility between replicates of each sample type was observed regardless the dataset used as explanatory factors. CAP ordinations using a range of PTEs (Co, Cu, Hg, Mn, Ni, Sb, Th, Pb, Zn) as explanatory factors, explained up to 78 % of the variance observed, of which 29% is explained in the first two axes (17% and 12%, respectively) (Fig. 4a). The sample type was the most explanatory variable (36.96%) followed by the Hg concentration (6.41%), both associated with the first axis. All the other variables (Mn, Co, Ni, Cu, Zn, Sb, Pb and Th) analyzed were not significant (Table S4). Likewise, CAP ordinations using physicochemical parameters and enzymatic activities as variables explained 73% of the variance observed (17% and 12% respectively for the first two axes) (Fig. 4b). None of the physicochemical parameters nor the enzymatic activities were significant explanatory variables of the composition of the bacterial community. For all CAP ordinations, reference- and contaminated-samples showed similar bacterial community composition ($p=0.734$) that was differing from those of tailings and dumps which were similar ($p=0.056$). The composition of the bacterial community of OMW was markedly different from that of all the other samples analyzed no matter the variables considered ($p<0.036$).

Taxonomic analysis of the 16S rRNA amplicon sequences at phylum level showed that the relative abundance of the major bacterial phyla was very similar in reference- and contaminated- samples. On the contrary, it markedly differed between reference-samples and dumps. All samples were dominated by bacteria belonging to Proteobacteria, Actinobacteria, Chloroflexi, Acidobacteria, Gemmatimonadetes, Bacteroidetes, Firmicutes, TM7, Verrucomicrobia and Cyanobacteria (Fig. 5). Proteobacteria, Actinobacteria, Chloroflexi, Acidobacteria, Firmicutes, TM7 and Cyanobacteria were observed in similar abundance in all the sample types ($p>0.05$). Gemmatimonadetes and Bacteroidetes were found in significantly lower amount in dumps than in reference-samples ($p<0.035$). Verrucomicrobia had

307 significantly lower relative abundances in dumps, tailings and OMW than in the reference-
308 samples ($p<0.024$).

309 To further identify the OTUs responsible of differences in the composition of the bacterial
310 community between samples, a correlation analysis was performed with OTUs represented at
311 least once in all four replicates of a given sample type (a total of 76 different OTUs) using the
312 diablo R package. Among these 76 OTUs, 57 responsible of the differences observed between
313 substrate types (Pearson's correlation > 0.4) were plotted in a heatmap (Fig 6). All
314 discriminant OTUs affiliated to 7 of the 10 most abundant bacterial phyla detected
315 (Actinobacteria, Chloroflexi, Acidobacteria, Bacteroidetes, Cyanobacteria,
316 Gemmatimonadetes and Proteobacteria).

317 Similar abundances were found in discriminant OTUs for reference- and contaminated-
318 samples, confirming the results from α-and β-diversity analyses. Microorganisms belonging
319 to the orders Actinomycetales, Sphingomonadales, Burkholderiales, Rhodospirillales,
320 Stramenopiles, RB41, Solibacterales, Ellin 329 and some microorganisms belonging to the
321 order Rhizobiales were highly represented in reference- and contaminated-samples.
322 Microorganisms belonging to the families *Acidobacteriaceae*, *Sphingobacteriaceae* and
323 *Xanthomonadaceae,* the order Solirubrobacterales, N1423WL, some Actinomycetales and
324 some Rhizobiales; and the genera *Sphingomonas* and *Segetibacter* were highly represented in
325 OMW but scarcely detected in reference- and contaminated-samples. Regarding the dumps,
326 five OTUs belonging to Acidomicrobiales, B12-WMSP1, *Acidobacteriaceae* and
327 *Enterobacteriaceae* phylotypes were found in high abundance. Finally, a high heterogeneity
328 was observed for the discriminant OTUs in tailings.

329

330 **4. Discussion**

Mining soils are generally rich in PTEs that are known to have ecotoxicological effects on soil microorganisms by disrupting their metabolism, affecting soil enzymatic activity and having consequence on the abundance, composition and diversity of soil microbial communities (Zhao et al., 2020). The analysis of the PTEs concentrations in the five different sample types allowed the classification of the samples as highly polluted (reference- and contaminated-samples) and very highly polluted (OMW, tailings and dumps). Although the potential value of OMW as fertilizer or metal immobilization amendment in polluted soils was shown in previous studies (Hmid et al., 2014; Paredes et al., 1999), here the application of OMW showed no remediation activity. It is noteworthy that a number of recent studies have shown the negative impact of OMW on aquatic and terrestrial ecosystems (Ntougias et al., 2013). Soil enzymes are mainly produced by microorganisms, which play a crucial role in nutrient cycling and consequently, in soil fertility (Adetunji et al., 2017). Microbial enzymes are highly sensitive to metals, and their use as standard biochemical indicators has been widely proposed to evaluate the quality of polluted soils (Pająk et al., 2018; Tang et al., 2019). However, several studies have showed no effect of PTEs contamination on some soil enzymatic activities, such as dehydrogenase, catalase, acid phosphomonoesterase and amylase (Campos et al., 2018a; Liu et al., 2020; Yang et al., 2016). This phenomenon could be due to three reasons: (I) the long-term presence of metals in the soil, which might promote the development of heavy metal resistant microorganisms (Ciarkowska et al., 2014); (II) variations in soil physicochemical properties, which may influence the activity of soil microbial communities (Xian et al., 2015), the bioavailability and speciation of the pollutants (Rieuwerts et al., 1998); and (III) antagonistic effects between various PTEs (Tang et al., 2019). Our results showed that enzyme activities were similar in reference- and contaminated-samples. This might be explained by the high organic matter content in these two sample types, which may on the one hand, provide nutrient source for the development of

microorganisms and the production of enzymes and on the other hand, reduce the bioavailability of metals and metalloids, limiting their toxicity towards microbial enzymatic activities (De Santiago-Martín et al., 2013; Lair et al., 2007). It is important to note, that organic matter can sometimes increase the mobility and bioavailability of pollutants (Lindsay, 1991; Meunier et al., 2011; Wang and Mulligan, 2006). Consequently, further research is needed to better understand the processes governing the complex interactions between organic matter and pollutants. Additionally, all enzymatic activities were very low in OMW as confirmed by the geometric mean index. The important amount of phenols with powerful antimicrobial properties and high content of PTEs, might explained the low enzymatic activities recorded in OMW samples (Siles et al., 2014). DHA activities measured in tailings and dumps were significantly lower than those of reference-samples. Keeping in mind the fact that the inhibition of DHA activity in response to PTEs such as Pb, Cu, Cd, Zn has been previously described (De Santiago-Martín et al., 2013; Pająk et al., 2018; Parelho et al., 2016; Tang et al., 2019), one could hypothesize that the high concentration of PTEs in tailings and dumps would explain the observed low enzyme activities. Similar results were found for ARS activity, that is a key player in the sulfur cycle and known to be inhibited by PTEs (De Santiago-Martín et al., 2013; Gülser and Erdoğan, 2008; Stefanowicz et al., 2020). No-matter the sample type, acid PhA activities were higher than those of alkaline PhA probably due to the acidic nature of all the samples (Adetunji et al., 2017). Acid PhA activity was lower in tailings than in the reference-samples which is in accordance with known sensitivities of these enzymes to heavy metals (Angelovičová et al., 2014; De Santiago-Martín et al., 2013; Gao et al., 2010). Despite the fact that β-Gal has been reported as a sensitive bioindicator of metal contamination of soils (De Santiago-Martín et al., 2013; Martínez-Iñigo et al., 2009), all samples showed similar β-Gal activities except in dumps where slightly lower activities were recorded.

As observed with microbial enzymatic activities, the exposure to PTEs can also affect key microbial processes, which reflects on changes in microbial composition and diversity. On the one hand, PTEs such as Pb, Zn or Hg can exert toxic effects compromising the survival and growth of specific microbial guilds (Abdu et al., 2017; Doelman and Haanstra, 1984). On the other hand, PTEs can promote the emergence of specific resistant microorganisms able to cope with the ecotoxicity of these contaminants (Gillard et al., 2019; Martinez et al., 2006; Sobolev and Begonia, 2008). Here, the lowest values of all the α-diversity indices (Chao1, PD whole tree and Simpson reciprocal) were recorded in dumps, probably as consequence of the very high concentrations of Pb, Zn, Sb, Co and Cu present in these samples and low organic matter. Indeed, the reduction in soil bacterial α-diversity as consequence of Pb, Zn, Sb, Co or Cu toxic effects has previously been reported (Luo et al., 2018; Nunes et al., 2016; Stefanowicz et al., 2008; Xie et al., 2016). Similarly, Chao1 and PD whole tree values were lower in tailings than in the reference-samples. It is noteworthy that PTEs concentrations in tailings were not significantly different from that of reference-samples, suggesting that differences observed in α-diversity may result from other physicochemical parameters governing the bioavailability of PTEs. Chao1 diversity indices in OMW samples were significantly lower than that of reference-samples. As observed for the enzymatic activities, the high content of organic matter rich in phenols in OMW could have ecotoxicological effects on survival and growth of phenol-sensitive microorganisms and therefore decreased the microbial diversity. In this regard, changes in the structure of ammonium oxidizers bacteria and Actinobacteria have been previously reported (Karpouzas et al., 2010; Mekki et al., 2006). All alpha diversity indices were similar in both reference- and contaminated-samples.

Multivariate β-diversity analysis based on Bray Curtis distance matrix confirmed the results observed from α-diversity: reference- and contaminated-samples clustered together no

406 matter the explanatory factors (PTEs, enzymatic activities or texture). These two samples
407 were clearly separated from tailings and dumps. OMW was discriminated from all others
408 whatever the factors considered. Besides the sample type, which explained 37% of the
409 variance observed, concentration of Hg was the only significant explanatory factor (6% of the
410 variance). In line with our work, the influence of soil properties and heavy metals on the
411 structure of bacterial communities has been previously showed in contaminated soils (Li et
412 al., 2017; Liu et al., 2018; Nunes et al., 2016; Tipayno et al., 2018)

413 Phylogenetic analysis revealed that the majority of bacteria from all samples affiliated to
414 the Proteobacteria, Actinobacteria, Chloroflexi, Acidobacteria, Gemmatimonadetes,
415 Bacteroidetes, Firmicutes, TM7, Verrucomicrobia and Cyanobacteria phylotypes, in line with
416 previous studies performed in a range of PTEs polluted soils (Chen et al., 2018; Guo et al.,
417 2017; Pacwa-Płociniczak et al., 2018; Tipayno et al., 2018; Zhen et al., 2019). Overall, their
418 relative abundances were similar in all samples, with the exception of the bacteria belonging
419 to Gemmatimonadetes, Bacteroidetes and Verrucomicrobia, whose relative abundance were
420 significantly lower in dumps as compared to reference-samples. A significant reduction in the
421 relative abundance of Verrucomicrobia was also observed in tailings and OMW. The
422 significantly higher concentrations of PTEs found in in tailings and OMW may explain the
423 reduction of these phylotypes. This assumption is in line with the reduction of
424 Verrucomicrobia previously reported in a study performed on an agricultural paddy soil
425 polluted with cadmium (Luo et al., 2019) and Yellow river sediments heavily polluted with
426 cadmium, arsenic, lead and mercury (Chen et al., 2018). Similarly, the relative abundance of
427 members of several phyla including Verrucomicrobia and Bacteroidetes was found to
428 significantly decrease in a long-term Cu polluted site (Berg et al., 2012). In this regard, our
429 findings are consistent with a recent study that proposes that changes in the response of some
430 bacterial groups (the Verrucomicrobia/Chlamydiae ratio) can be used as bioindicators of

heavy metal pollution (Schneider et al., 2017). Contrary to our results, studies from a metal polluted forest soil showed an increase in the Gemmatimonadetes (Azarbad et al., 2015) but a negative correlation between the relative abundance of Verrucomicrobia and pollution levels as observed in our study.

Further analysis at lower taxonomical level led to the identification of 57 OTUs responsible of α- and β- diversity changes observed between different sample types. Discriminant OTUs were found in similar abundances in reference- and contaminated-samples. Interestingly, OTUs barely found in reference- and contaminated-samples were significantly more abundant in OMW, suggesting that these OTUs may be able to grow in a PTEs contaminated environment rich in organic matter. OTUs related to the B12-WMSP1 group described by Costello and Schmidt (Costello and Schmidt, 2006) in fumarolic soils (Costello et al., 2009; Schmidt et al., 2018; Tebo et al., 2015) were found in higher amount in dumps than in the reference-samples.

5. Conclusions

The importance of soil health and quality in ecosystem services underlines the need to understand the processes governing the behavior and effects of PTEs on soil microorganisms and supported functions. This assumption is especially relevant in highly polluted areas such as mining areas. Here, we evaluated the degree of contamination in a long-term mining site and assessed the ecotoxicological effects of PTEs on bacterial communities. We observed that low PTEs concentrations can have a strong impact on the diversity and composition of microbial communities while physicochemical parameters such as organic content can either help counteract or enhance their negative effects. Understanding the relationship between physicochemical parameters and diversity and composition of microorganisms can contribute

455 to the environmental risk assessment of long-term polluted sites and help create strategies to
456 restore contaminated sites and mitigate the pollution dissemination.

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464 characterization.

465 **ABBREVIATIONS**

466 ARS, Arylsulfatase enzyme activity

467 DHA, Dehydrogenase enzyme activity

468 EC, Electric conductivity

469 OMW, Olive-oil mill waste

470 OM, organic matter

471 PhA, Phosphomonoesterase enzyme activity

472 PTEs, Potentially toxic elements

473 β-Gal, β-galactosidase enzyme activity

474

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Figures

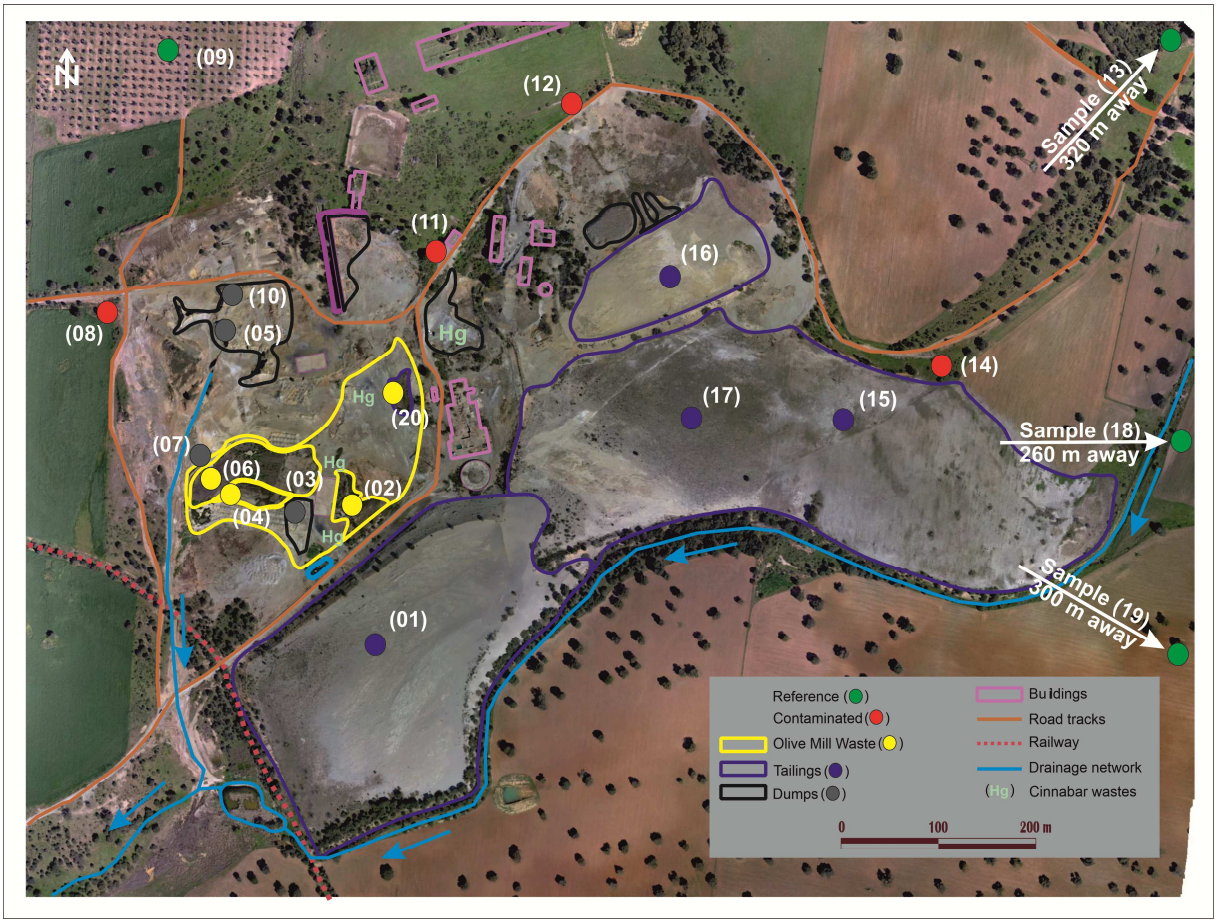


Figure 1. Geographical area and sampling sites (reference, green; contaminated, red; olive mill waste, yellow; tailings, blue and dumps, grey) in the study area.

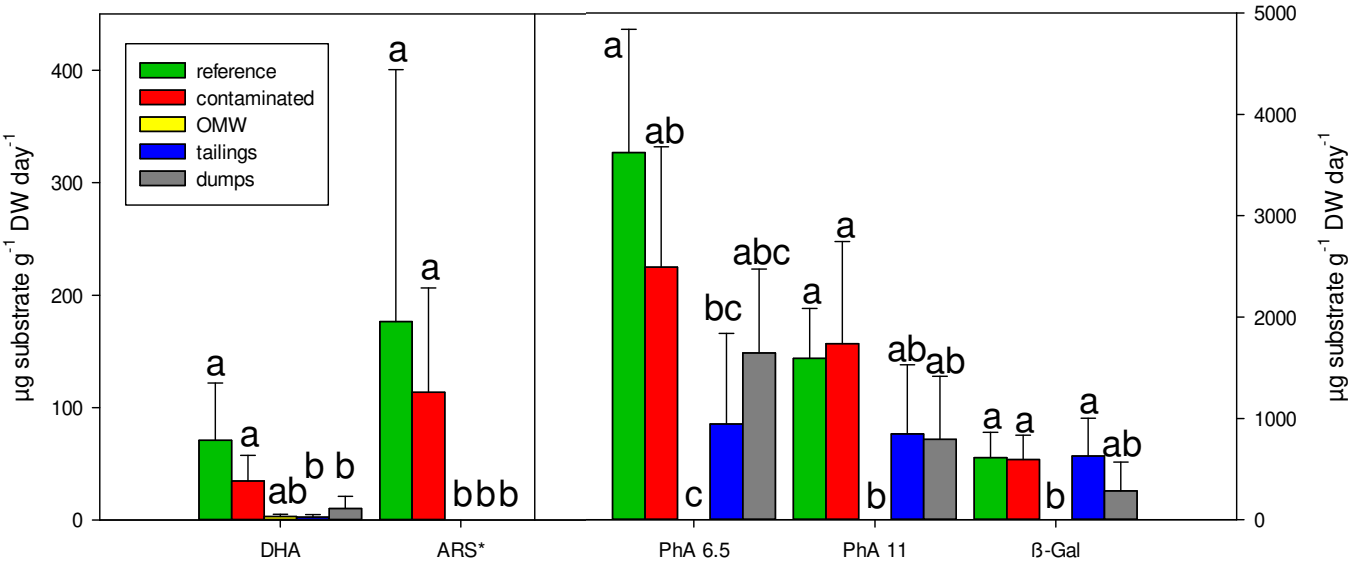


Figure 2. Enzymatic activities (mean ± S.D): dehydrogenase (DHA) (µg TPF g^{-1} DW day^{-1}), arylsulfatase (ARS) (µg PNF g^{-1} DW day^{-1}), acid phosphomonoesterase (PhA 6.5) (µg PNF g^{-1} DW day^{-1}), alkaline phosphomonosterase (PhA 11) (µg PNF g^{-1} DW day^{-1}) and β-Galactosidase (β-Gal) (µg PNF g^{-1} DW day^{-1}) for reference-, contaminated-samples, olive mill waste (OMW), tailings and dumps. ANOVA followed by Tukey's test and Kruskal Wallis tests (*) were performed. For DHA, ANOVA was performed on log-transformed data. For each parameter, different letters within the same line indicate significant differences ($p < 0.05$). n=4

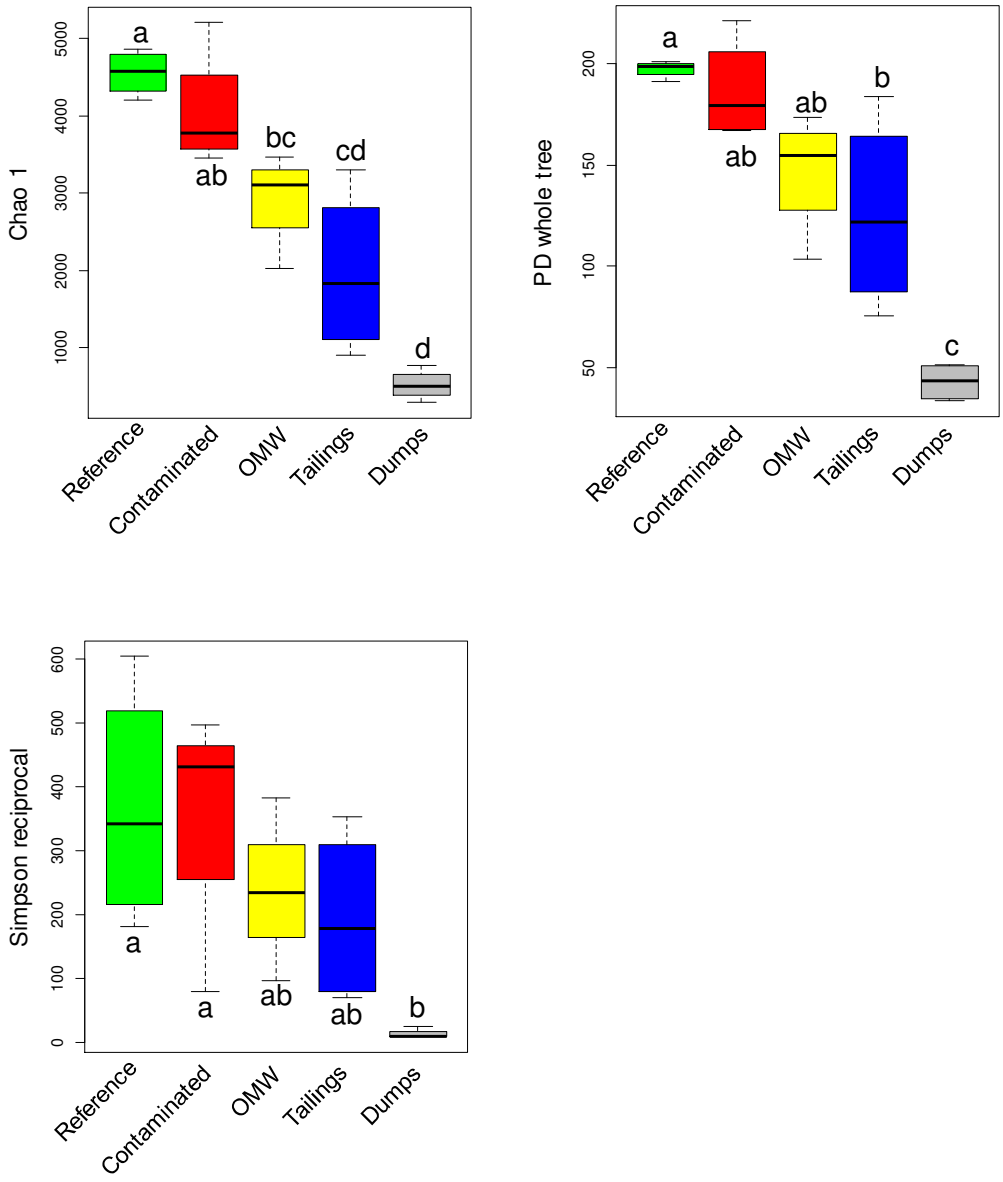
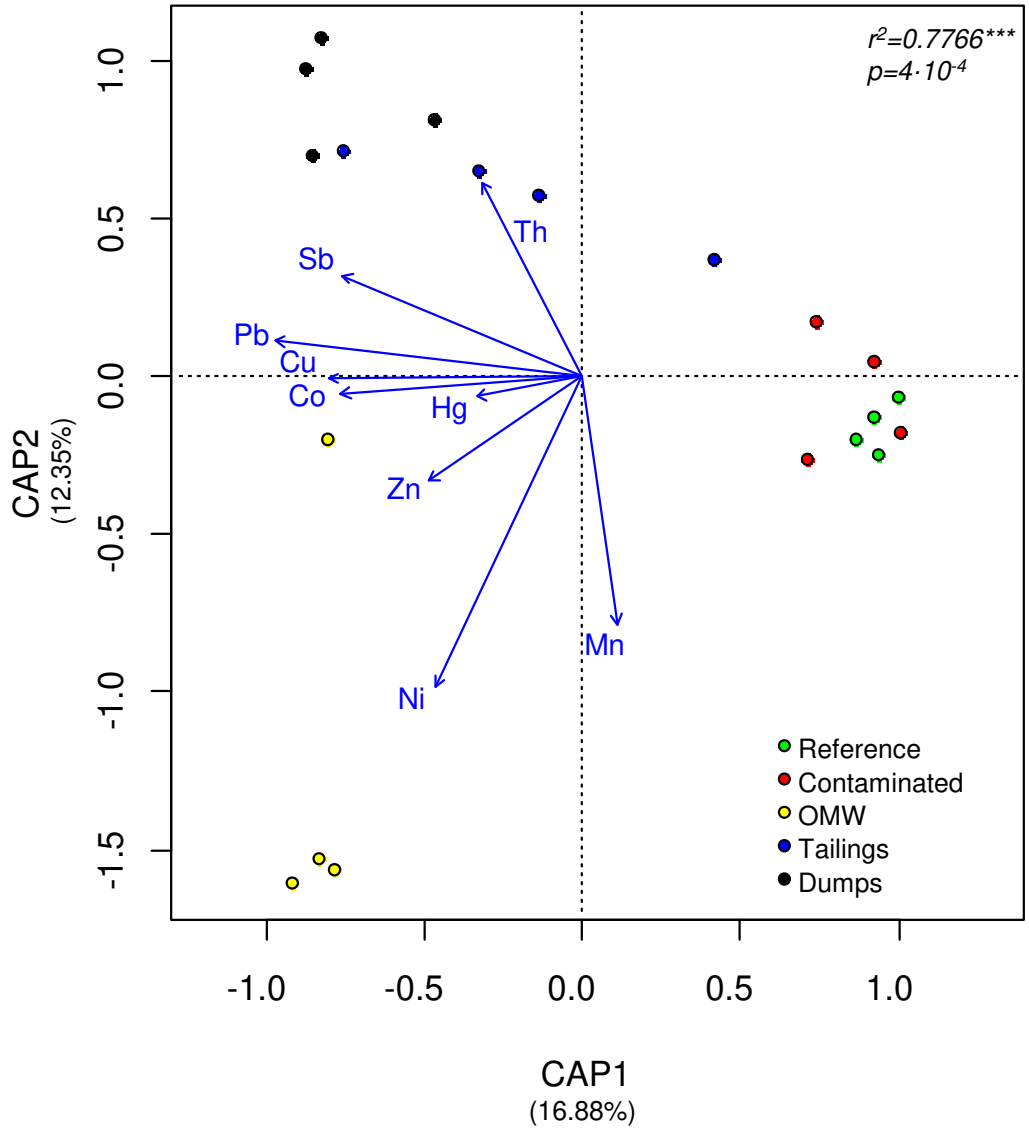
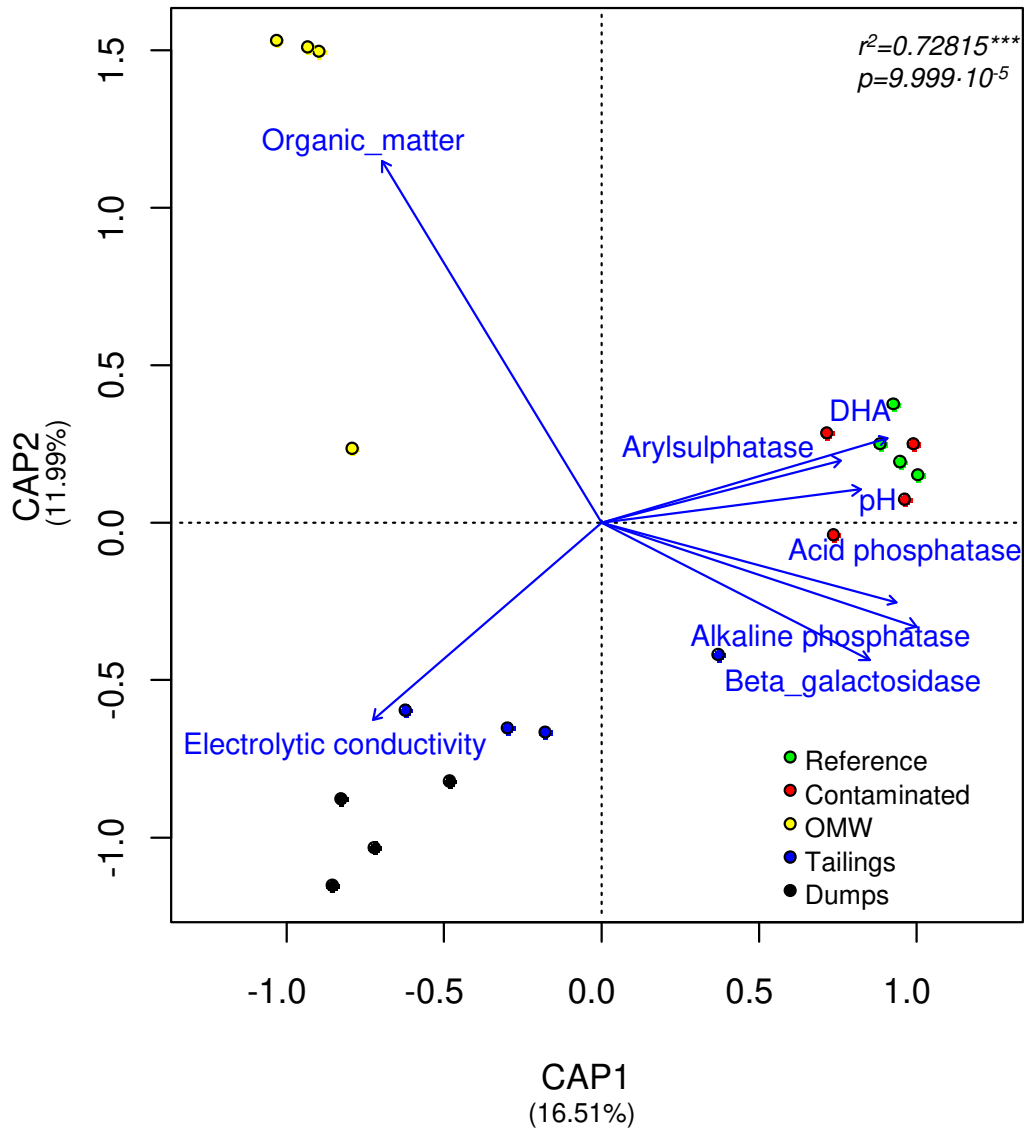


Figure 3. Bacterial α-diversity indices (mean values ± standard deviation) derived from amplicon sequencing of the 16S rDNA gene copies from reference-, contaminated-samples, olive mill waste (OMW), tailings and dumps. ANOVA test followed by Tukey's test was performed. For each diversity index value, different letters indicate that samples are significantly different for each treatment and time. For dominance, ANOVA was performed on log-transformed data. n=4


r^2=0.7766***
p=4·10^{-4}
Th
Sb
Pb
Cu
Co
Hg
Zn
Mn
Ni
Reference
Contaminated
OMW
Tailings
Dumps
CAP2
(12.35%)
CAP1
(16.88%)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-1.0
-0.5
0.0
0.5
1.0

a



b

Figure 4. Bacterial β-diversity analysis for reference-, contaminated-samples, olive mill waste (OMW), tailings and dumps using Bray Curtis distance matrix of 16S rRNA sequences and (a) PTEs concentrations or (b) enzymatic analysis as explanatory factors. The variance explained by each axis is given as percentage. For each sample type, the four replicates are represented with the same color.

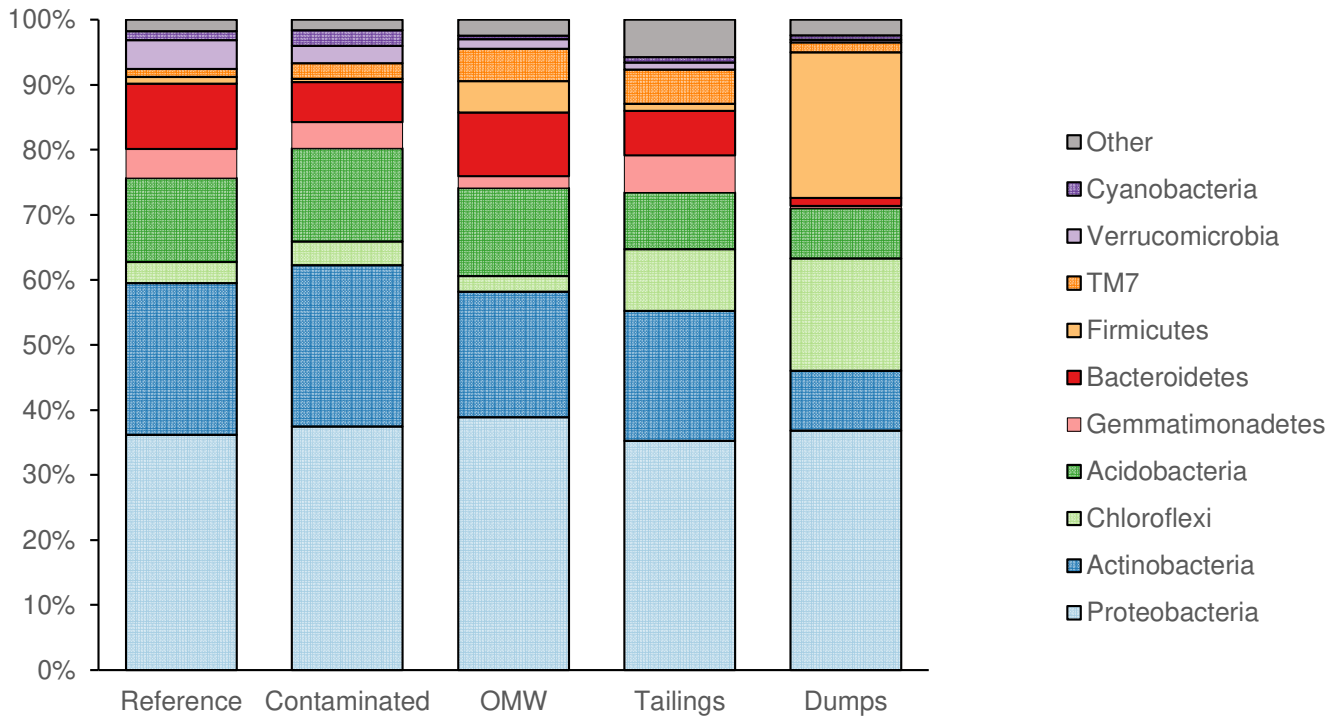


Figure 5. Relative abundance of the major bacterial phyla (expressed as % of the total number of OTUs) for reference-, contaminated-samples, olive mill waste (OMW), tailings and dumps. Phyla whose relative abundance was below 5% were grouped as "others". ANOVA followed by Tukey's test and Kruskal Wallis tests (Proteobacteria, Chloroflexi, Acidobacteria and Cyanobacteria) were performed. For Firmicutes and TM7, ANOVA was performed on log-transformed data. n=4

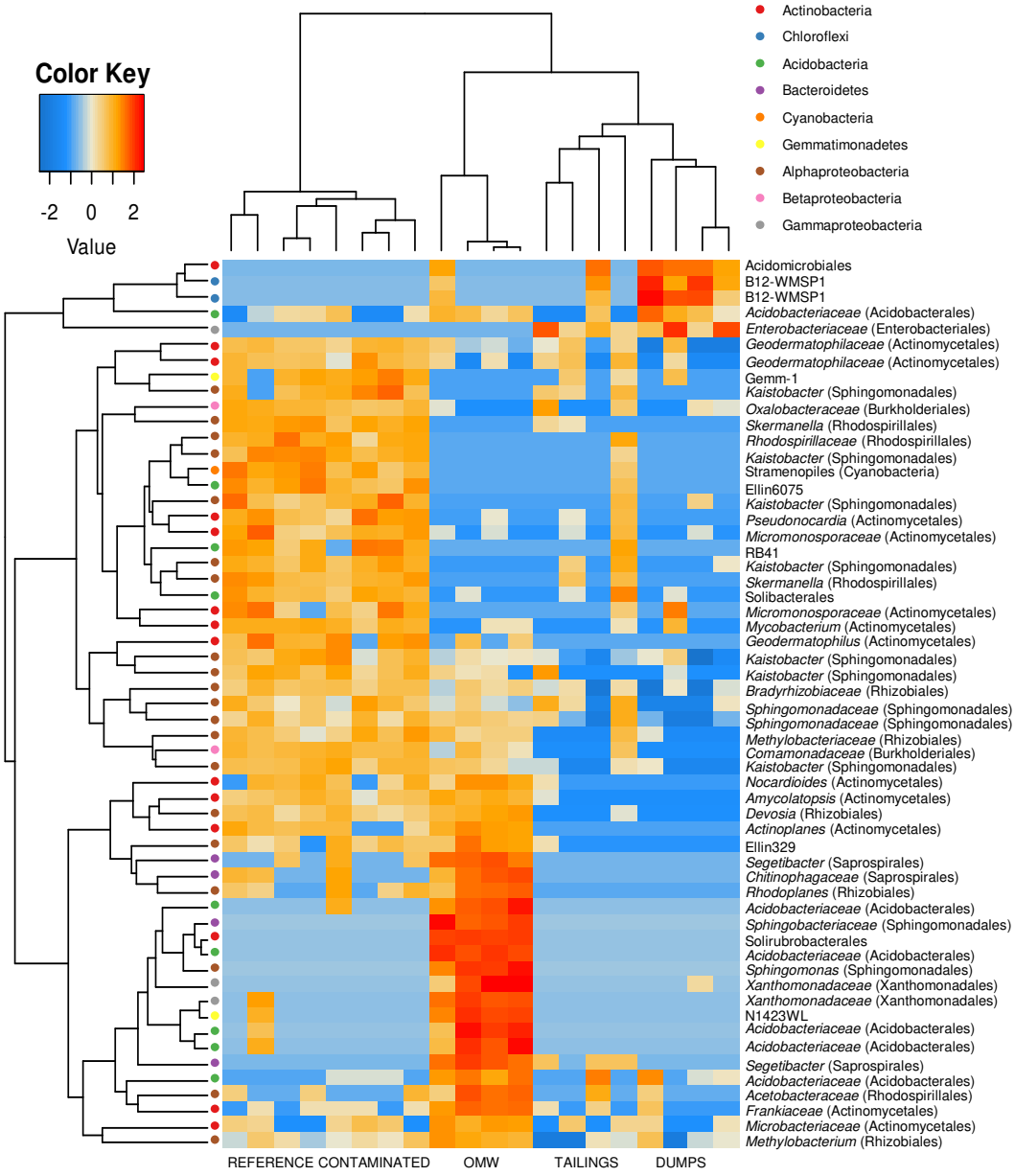


Figure 6. Heatmap plot representing relative abundances of OTUs correlated to physicochemical and enzymatic analysis (correlation cut-off r>0.4) in reference-, contaminated-samples, olive mill waste, tailings and dumps.

Tables

Table 1. Distribution (geographic coordinates) and main characteristics of samples

Sample type	Sample	X (UTM)	Y (UTM)	Altitude	Other Characteristics
Reference	SQD-9	388986	4298003	666	Recently fertilized
Reference	SQD-13	390156	4298068	664	
Reference	SQD-18	390256	4297642	671	Dry land, plowed
Reference	SQD-19	390207	4297137	669	Fallow, unplowed
Contaminated	SQD-8	388935	4297593	687	Agricultural
Contaminated	SQD-11	389261	4297675	675	2 meters from a plant
Contaminated	SQD-12	389392	4297805	678	Developed soil
Contaminated	SQD-14	389798	4297560	663	
Olive Mill Waste	SQD-2	389170	4297396	655	
Olive Mill Waste	SQD-4	389103	4297409	659	
Olive Mill Waste	SQD-6	389059	4297448	657	
Olive Mill Waste	SQD-20	389238	4297521	671	With visible dump debris
Tailings	SQD-1	389208	4297287	646	Aeolic erosion
Tailing	SQD-15	389704	4297503	667	
Tailing	SQD-16	389521	4297623	667	
Tailing	SQD-17	389553	4297525	673	
Dumps	SQD-3	389129	4297398	658	Heterogeneous granulometry
Dumps	SQD-5	389081	4297558	658	Heterogeneous granulometry
Dumps	SQD-7	389033	4297470	662	Flooded area
Dumps	SQD-10	389032	4297607	674	Heterogeneous granulometry

Table 2. Physicochemical characteristics of reference-, contaminated-samples, olive mill waste, tailings and dumps. Values were mean ± standard deviation. ANOVA followed by Tukey's test were performed. For EC and OM, ANOVA was performed on log-transformed data. For each parameter, different letters within the same line indicate significant differences ($p < 0.05$). For EC and OM, ANOVA performed on log-transformed data. n=4

	Reference	**Contaminated**	**Olive Mill Waste**	**Tailings**	**Dumps**
pH	6.1 ± 0.3a	6.8 ± 1.0a	5.2 ± 0.6ab	6.7 ± 1.7a	3.9 ± 0.5b
EC (mV)	62.4 ± 27.8a	90.6 ± 49.9a	651.3 ± 489.7ab	415.6 ± 378.8ab	2282.3 ± 1786.4b
OM (%)	2.9 ± 1.9a	8.1 ± 8.9a	60.6 ± 19.7b	2.0 ± 0.4a	1.6 ± 0.5a
Sample type	sandy loam	sandy loam	loamy sand	silt loam	sandy loam

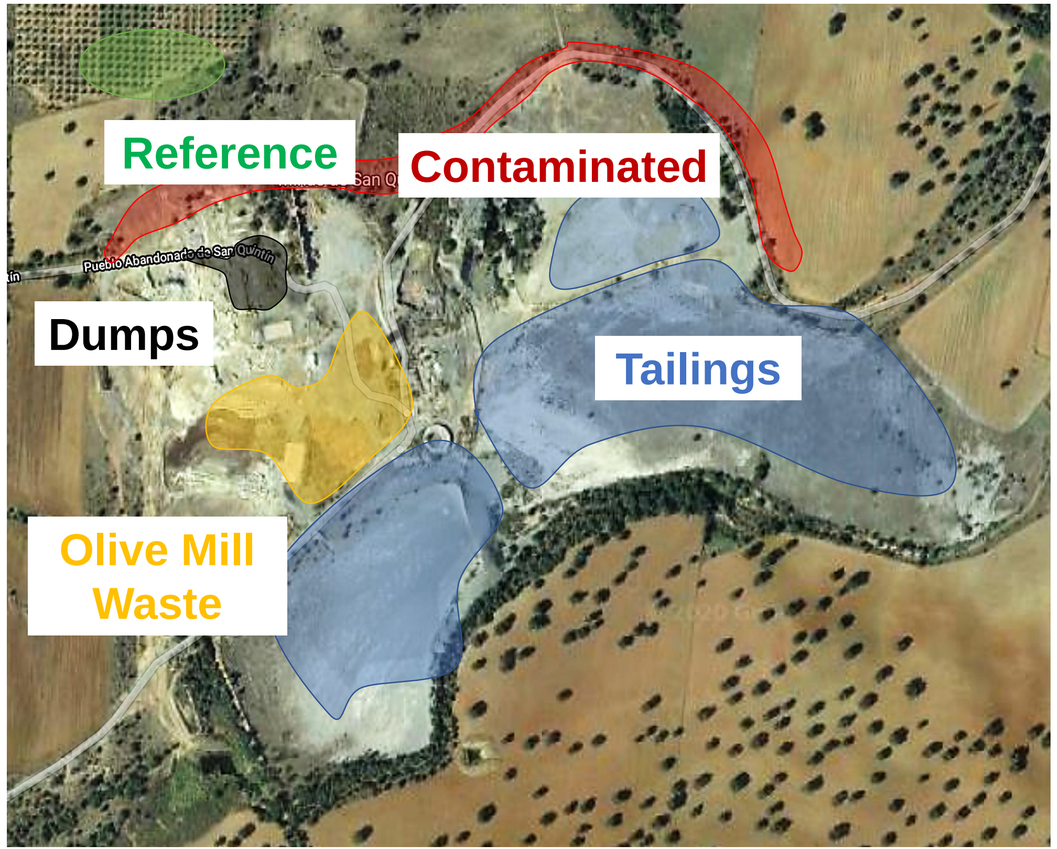
EC: electric conductivity, OM: organic matter

Table 3. PTEs concentrations (mg/kg) (mean values ± standard deviation) and Pollution Load Index (PLI) in reference-, contaminated-samples, olive mill waste, tailings and dumps. ANOVA test followed by Tukey's test and Kruskal Wallis followed by pairwise comparisons with Bonferroni correction (*) were performed. Values indicated by different letters are significantly different within the same line. For Ni and Cu ANOVA was performed on root square transformed data. For Sb, ANOVA was performed on inverse transformed data, respectively. For Zn, Hg and Th, ANOVA performed on log-transformed data, respectively. n=4

	Reference	Contaminated	Olive Mill Waste	Tailings	Dumps
Pb	315.8 ± 332.6a	3696.5 ± 4406.2a	38890 ± 20687.0b	4030.2 ± 7170.8a	48597.5 ± 16835.1b
Zn	204.8 ± 153.8a	1522.4 ± 2165.2ab	86050 ± 99094.2c	2024.5 ± 2492.1ab	4640.0 ± 2259.5b
Hg	0 ± 0a	55.4 ± 92.1a	3605.8 ± 6752.0a	557.1 ± 430.8a	130.8 ± 142.5a
Sb	16.8 ± 1.8a	37.4 ± 31.0abc	330.9 ± 435.2bc	65.5 ± 87.7ab	433.6 ± 198.9c
Co	221.7 ± 76.4a	268.3 ± 80.0ab	451.7 ± 78.4bc	192.5 ± 27.9a	503.9 ± 180.6c
Cu	17.3 ± 10.0a	165.6 ± 297.6a	963.7 ± 466.2b	113.4 ± 196.7a	1000.6 ± 805.0b
Mn*	688.2 ± 238.9ab	1572.5 ± 908.6a	1637.4 ± 1866.4ab	423.5 ± 136.5ab	210.4 ± 90.8b
Ni	31.4 ± 18.7a	38.9 ± 31.4a	132.1 ± 95.6a	37.0 ± 14.0a	27.6 ± 8.1a
Th	9.7 ± 3.9ab	4.6 ± 4.1ab	0 ± 0b	3.8 ± 6.9ab	35.1 ± 23.7a
PLI	1.0 ± 0.1	3.2 ± 2.8	21.1 ± 23.9	10.5 ± 4.8	7.4 ± 2.4

PLI: Pollution Load Index

SAN QUINTÍN MINE



Analytical chemistry

Microbial enzyme activities

Composition and diversity of bacterial community

Ecotoxicological risk assessment of San Quintín mine area