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Do metal contamination and plant species affect microbial abundance and bacterial diversity in the rhizosphere of metallophytes growing in mining areas in a semiarid climate?

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Abstract

Purpose Mining areas are low-quality habitats for macro- and microorganisms' development, mainly due to the degradation of the soil quality by metal pollution. The present work aimed to analyze the influence of metal contamination and of plant species on the rhizospheric microbial communities of four indigenous metallophytes (*Ononis natrix*, *Haloxylon scoparium, Peganum harmala*, and *Aizoon canariense*) growing along a metal contamination gradient in Kettara mine near Marrakech, Morocco.

Materials and methods In pyrrhotite mining areas (Kettara mine, Morocco), rhizosphere soil samples were collected from four predominant indigenous metallophytes (*O. natrix*, *H. scoparium*, *P. harmala*, and *A. canariense*) growing along a metal contamination gradient (ZC, control zone; Z1, high metal contamination; Z2, moderate metal contamination; Z3, low metal contamination). Microbial communities were analyzed by using microbial counts and by denaturing gradient gel electrophoresis (DGGE). The physicochemical properties (pH, conductivity, total organic carbon, nitrogen, P Olsen, and metal concentrations) of soils were also determined.

Results and discussion The physicochemical analysis revealed that rhizospheric soils from Z1, Z2, and Z3 were relatively poor in nutrients as they presented low levels of total organic carbon and nitrogen, organic matter and available P. Moreover, these rhizospheric soils showed high concentrations of metals, especially Cu and Pb, which significantly reduced the abundance of the different groups of soil microorganisms (bacteria, fungi, and actinomycetes) and the activity of soil dehydrogenase. The analysis of bacterial communities by DGGE revealed that bacterial diversity was not negatively affected by metal contamination being higher in the most contaminated area (Z1).

Conclusions Overall, the microbial abundance, the composition, and the diversity of rhizospheric bacterial communities were more influenced by the environmental factors in sampling zones than by plant cover. Microbial counts and enzymatic activity were both systematically affected throughout the metal gradient, evidencing as good indicators of the harmful effects of anthropogenic disturbances in soils. *H. scorparium* and *P. harmala* proved to be good candidates for the development of phytotechnological programs aiming the revegetation of mining degraded areas.

Keywords Bacterial diversity · Denaturing gradient gel electrophoresis (DGGE) · Metal pollution · Microbial counts · Mine soil · Rhizosphere

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1 Introduction

Soil contamination by anthropogenic activities has widely increased in last decades. Among soil pollutants, there is a particular concern with metals, as most of them cannot be transformed into less toxic species, persisting indefinitely in the environment (Ali et al. 2013; Ayangbenro and Babalola 2017). In addition to their harmful effects on plants, animals, and soil biodiversity, metals can also compromise human health, being a cross-cutting problem to several countries (Science Communication Unit 2013).

Mining industry is one of the most polluting activities in the world constituting a permanent threat for ecosystems and public health (Li et al. 2014a). Mining operations (e.g., milling, disposal of tailings) generate large amounts of toxic materials, including metals, which can be easily diffused into the surrounding environment (El Khalil et al. 2008; Mokhtari et al. 2018), even after the end of mining works (Acosta et al. 2011). The high concentrations of metals and the harsh soil conditions (e.g., low pH and organic matter content, erosion) registered in the vicinity of mining areas significantly impair plant survival and development, resulting in a scarce vegetation cover in these areas, which increase the risk of wind and hydraulic dispersion of contaminated particles (Monterroso et al. 2014).

Metallophytes are plants able to grow in highly metal-contaminated soils as they have developed mechanisms to tackle the harmful effects of metals in their tissues (Alford et al. 2010). Several studies have shown the potential of native metallophytes present in many metalliferous sites to be used in phytotechnological approaches for the remediation and revegetation of environmental degraded areas (Whiting et al. 2004; Nadgórska-Socha et al. 2015). In Kettara mine, despite the relatively high levels of metals in the soils, most metallophyte species growing in the area present low levels of metals in their shoots (Boularbah et al. 2006a). As such, according to the criteria of Baker and Brooks (1989), the indigenous species of Kettara mine should be classified as hypertolerant but not as hyperaccumulators, being good candidates for phytostabilization strategies.

The microorganisms present in the rhizosphere of these metallophytes have a crucial role on the speciation and bioavailability of metals in soil (Li and Wong 2011), while they can improve plant growth and their establishment in metal-enriched soils (Moreira et al. 2016a, 2016b). Metals are known to adversely affect the abundance, activity, and diversity of soil microbial communities (Yuangen et al. 2006; Khan et al. 2010). As such, these biological parameters have been used as indicators of soil quality and health, since they are known to be very sensitive to anthropogenic soil disturbances (Alkorta et al. 2003).

The composition of the rhizospheric microbial communities may be shaped by several factors, including soil physicochemical properties, environmental stresses, and type of vegetation cover (Berg and Smalla 2009; Li et al. 2014b). However, several authors also consider the plant species the key factor influencing the composition and abundance of rhizospheric microbial communities (Wieland et al. 2001; Steinauer et al. 2016), being the amount and type of root exudates released by the different plant species the driving force behind these changes (Berg and Smalla 2009). Increasing the knowledge on the response of microorganisms inhabiting the rhizosphere of metallophytes to metal pollution is an important stepping stone for the development of microbial-assisted phytoremediation solutions.

The present work aimed (i) to evaluate the effect of metal contamination and of the plant species colonizing the Kettara mining area on the microbial abundance and on the rhizospheric bacterial community composition and diversity; (ii) to assess the potential use of soil microbial parameters as soil quality indicators, and (iii) to assess the potential of native metallophytes to be used in phytoremediation strategies in mining affected areas.

2 Materials and methods

2.1 Site description and sampling

The present study was conducted in an abandoned pyrrhotite mine located in Kettara village, approximately 32 km northwest of Marrakech (31° 52' 15" N 8° 10' 31" W). As a result of more than 30 years of mining and milling operations in Kettara mine, more than 3 million tons of mine waste have been deposited over an area of approximately 16 ha (Hakkou et al. 2008).

Following a gradient of metal contamination previously defined (Boularbah et al. 2006a; Benidire et al. 2016), three sampling sites located at different distances from tailings ponds were chosen: Zone 1 (Z1), high metal contamination; zone 2 (Z2), moderate metal contamination; and zone 3 (Z3), low metal contamination (Fig. 1). A site located upstream of the mining area, 10 km away from the source of pollution, was used as control (ZC). Mine tailings were also sampled for physicochemical and microbiological analysis.

Four metallophyte species were commonly found in the selected zones—*Ononis natrix*, *Haloxylon scoparium*, *Peganum harmala*, and *Aizoon canariense* (Boularbah et al. 2006b). All plant species were found in ZC and Z2, while *A. canariense* and *O. natrix* were absent in Z1 and *P. harmala* and *A. canariense* were absent in Z3. A total absence of vegetation was noted in mine tailings.

Three entire plants were collected in spring, from the three sampling zones as well as from the control site. Plants were placed in sterile plastic bags and kept in a cooler until the laboratory. The rhizospheric soil was
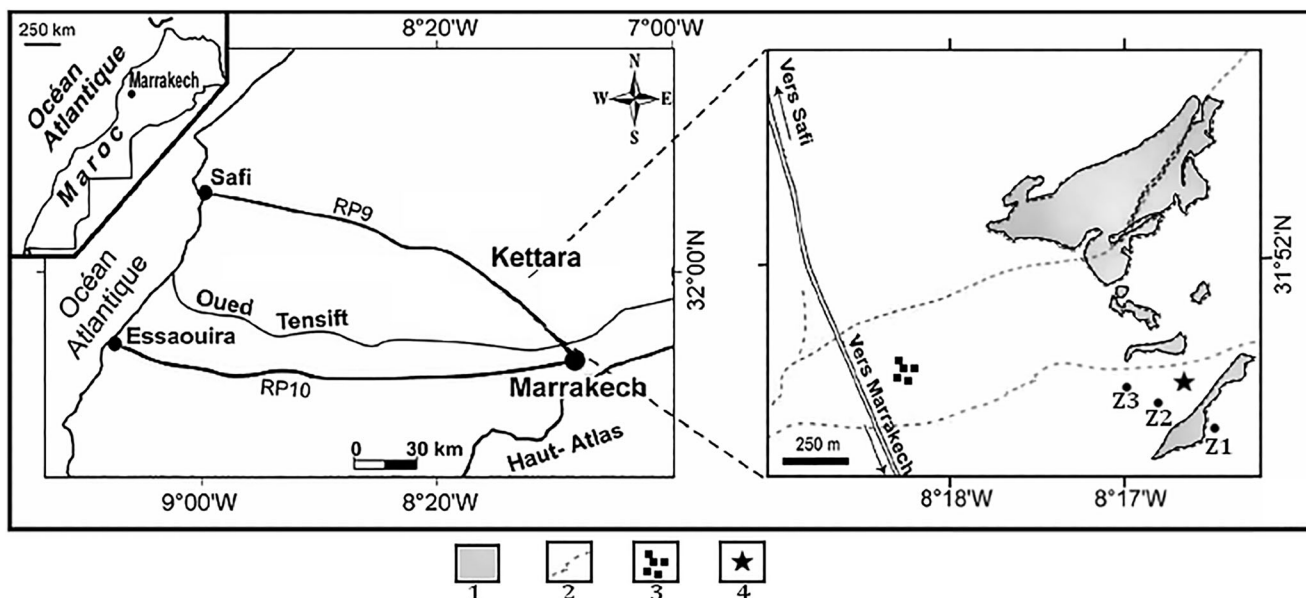
Fig. 1 Kettara mine location. (1) Tailings; (2) Talweg; (3) Kettara village; (4) Kettara mine: sampling zones (Z1, high metal contamination; Z2, moderate metal contamination; Z3, low metal contamination)

obtained by shaking the roots and a composite sample was made for each plant of each zone. Subsequently, the obtained soils were sieved (< 2 mm) and separated into different portions. The first portion was air-dried at room temperature for soil physicochemical analyzes, while the second one was stored in sterile plastic bags at 4 °C and assayed within 48 h for microbiological and enzymatic analyzes. The remaining soil was stored at − 20 °C for denaturing gradient gel electrophoresis (DGGE) analysis.

2.2 Soil and mine tailing physicochemical characterization

Soil pH and electrical conductivity (EC) were measured in H_2O using 1:2.5 and 1:5 soil:solution ratio, respectively. Total organic carbon (TOC) was determined using dichromate oxidation method (Blakemore et al. 1972), while soil total nitrogen (TN) and available phosphorus (P Olsen) content were determined using Kjeldahl and molybdenum blue method (Olsen and Sommers 1982), respectively. The extractable fraction of metals was determined using 0.01 M $CaCl_2$ as extractant. Total metal concentrations were determined after digestion of the soil with H_2SO_4 and HCl according to standard methods (NF EN ISO 11 466, 1995). The concentrations of metals were determined by flame atomic absorption spectrometry (Thermo Scientific iCE 3000) in the Laboratory of Hydrobiology, Ecotoxicology, Sanitation and Global Changes (FSSM, Marrakech, Morocco). All measurements were carried out in triplicate.

2.3 Germination test

In order to evaluate the phytotoxicity of the rhizospheric soils collected from each metallophyte, a germination test was carried out according to the AFNOR X 31-201 protocol (AFNOR 1986). The germination test was performed with seeds of common wheat var. Achtar (Sonacos) and lettuce var. Soleilan (Syngenta) due to their high sensitivity to chemical compounds, as recommended in different guidelines (EPA 1996; OECD 2006). Forty seeds of each plant species were placed in Petri dishes containing 40 g of soil and incubated for 7 days in darkness at 28 °C and 50% relative humidity in environmental chambers. A control was prepared using Petri dishes with Whatman paper moistened with distilled water instead of soil. The germination rate (G) was calculated according to the following formula:

$$G = \frac{B}{A} *100$$

Where A is the mean seed germination in the control test, and B is the mean seed germination in the tested soil.

2.4 Soil microbiological parameters

2.4.1 Dehydrogenase activity

Dehydrogenase activity (DHA) activity was performed according to Thalmann (1968). Briefly, 5 g of fresh soil was incubated with 5 ml of 2,3,5-triphenyltetrazoliumchloride (TTC) in 1 M Tris–HCl buffer (pH 7.5) for 24 h at 30 °C. The triphenyl formazan (TPF) produced was extracted with

40 ml of acetone. The suspension was filtered and the optical density of the supernatant was measured at 546 nm (UV-visible spectrometer, UV-3100PC Model).

2.4.2 Enumeration of soil culturable microorganisms

The number of culturable heterotrophic bacteria, fungi, and actinomycetes in the rhizosphere of each plant species and in mine tailings was determined by plating serial dilutions. Tryptone soy agar (TSA; Fluka) and potato dextrose agar (PDA; Difco) media were used for neutrophilic heterotrophic bacteria and fungi enumeration, respectively, since they are non-selective media and widely used for microbial enumeration in environmental samples (Aboudrar et al. 2007; Pereira et al. 2015; Naylo et al. 2019). The International Streptomyces Project 2 (ISP2) media was used for actinomycetes as it is the recommended media for the isolation and cultivation of actinobacteria (Shirling and Gottlieb 1966). Plates were incubated at 25–30 °C for 5 days. Data from triplicate counts were expressed as colony-forming units (CFU) g^{-1} dry soil.

2.4.3 Denaturing gradient gel electrophoresis

DNA was extracted from each rhizospheric soil composite sample using the Power Soil DNA Isolation Kit (MOBIO Laboratory, Inc., USA) according to the manufacturer's protocol. DNA was stored at − 20 °C.

In order to increase sensitivity and to facilitate the denaturing gradient gel electrophoresis (DGGE) analyses, a nested PCR technique was applied. The first round was performed with the universal primers 27F (5′-GAGTTGATCCTGGC TCAG-3′) and 1492R (5′-ACCTTGTTACGACTT-3′) (NZYTech) to amplify the bacterial 16S rDNA gene (Lane 1991), and the second PCR was carried out using the primers 338F-GC (50-GACTCCTACGGGAGGCAGCAG-30) and 518R (50-ATTACCGCGGCTGCTGG-30) (NYZTech) to amplify the V3 variable region of the 16S rRNA gene (Muyzer et al. 1993). The reactions mixtures and the PCR conditions were performed according to the protocol described in Pereira and Castro (2014). A slight modification was made in the first amplification, where it was used 1.25 μl of DMSO instead of 0.4 mg ml^{-1} of bovine serum albumin. All PCR products were verified in a 1.5% agarose gel in 1x TAE buffer.

DGGE was carried out in a Bio-Rad Dcode^{TM} Universal Mutation Detection System (Bio-Rad, USA). The nest-PCR products containing ca. 900 ng of DNA were loaded onto 8% (w/v) polyacrylamide gels (37.5:1, acrylamide/bisacrylamide) using a denaturing gradient from 35% to 60% of urea and formamide. After 16 h of migration at 70 V in 1x TAE buffer at a constant temperature of 60 °C, the gels were stained with a 5x Gel-Green Nucleic Acid Stain solution (Biotium, Inc.) for 10 min. DGGE images were obtained using a Safe Imager^{TM}

blue light transilluminator (Invitrogen^{TM}) and a microDOC gel documentation system (Cleaver Scientific Ltd.).

2.5 Metal concentrations in shoots of metallophytes

Metal concentrations in shoots of the metallophytes were determined as described by El Hamiani et al. (2010). Briefly, shoots were thoroughly cleaned with pressurized tap water followed by ultrapure water and air-dried at room temperature. The aboveground tissues were ground and stored in polythene bags at room temperature until analysis. Samples (0.5 g) were mixed with 8 ml of HNO$_3$ and 2 ml of H$_2$O$_2$ and placed in a hot plate at 100 °C until the total evaporation of the extractant under a fume hood. The mineralized material was recovered by the addition of ultrapure water and filtered with an ash-less filter. The volume was adjusted to 25 ml with ultrapure water and acidified with 1% HNO$_3$. The concentrations of metals in shoots were determined by flame atomic absorption spectrometry (Thermo Scientific iCE 3000).

Bioconcentration (BCF) factor was calculated as follows:

$$Bioconcentration\ Factor\ (\text{BCF}) = \frac{C\ tissue}{C\ soil}$$

where C tissue is the concentration of the target metal in the plant harvested tissue and C soil is the concentration of the same metal in soil (Ali et al. 2013).

2.6 Data analysis

Statistical analysis was carried out with the program SPSS (IBM, Armonk, NY, USA, version 25.0.) Results were analyzed by ANOVA, considering treatments as the independent variable. Significant statistical differences were established by Student-Newman-Keuls' test ($P < 0.05$). A correlation matrix between all physicochemical and microbiological parameters was calculated. The significance level reported ($P < 0.01$ and $P < 0.05$) is based on Pearson's coefficients.

DGGE profiles were analyzed with the Bionumerics software (version 6.6, Applied Maths, Saint-Martens, Belgium). Based on the presence or absence of individual bands, a binary matrix was constructed. A dendrogram was generated using the unweight pair group mean average (UPGMA) cluster analysis.

Species richness (S) was calculated based on the total number of distinct bands in a lane. The Shannon index (H) (Shannon and Weaver 1949) was determined by using the intensity of the DGGE bands given by peak heights in the densitometric curve, as follows:

$$H = -\Sigma \left(\frac{\text{ni}}{\text{N}}\right) \log \left(\frac{\text{ni}}{\text{N}}\right)$$

where n_i is the height of the peak and N is the sum of all peak heights of the densitometric curve for all bands in a given sample (used as estimates of species abundance). Pearson correlation analysis was also used to evaluate the relationships among α-diversity and soil microbiological and physicochemical properties by using SPSS (version 25.0).

The influence of sampling zones, plants, or their interactions on the composition of bacterial communities was evaluated through permutational multivariate analysis of variance (PERMANOVA) with 999 permutations using the *adonis* function from *vegan* package in R version 3.5.1 (R Core Team 2018). PERMANOVA was performed in Jaccard distances matrixes constructed from the DGGE dataset. Information in the Jaccard dissimilarity matrix was then graphically assessed with non-metric multidimensional scaling (NMDS) using *metaMDS* function (from *vegan* package in R) to create the NMDS ordination. The environmental variables were normalized using the function scale and their corresponding vectors were found and fitted in the NMDS ordination using the function *envfit* (from *vegan* package in R).

3 Results

3.1 Physicochemical characterization of rhizospheric soils and mine tailings

The physicochemical properties of mine tailings and of rhizospheric soils of the metallophytes species collected at Kettara mine are summarized in Tables 1 and 2. Mine tailings were extremely acid (2.8 ± 0.02) and showed high conductivity's values (2.45 ± 0.04). Soil rhizospheric pH varied between sampling zones and plant species ranging between 4.49 ± 0.03 in the rhizosphere of *H. scoparium* in Z1 and 8.17 ± 0.03 in the rhizosphere of *P. harmala* in ZC. In ZC, the rhizosphere of metallophytes was slight alkaline, while in Z1 and Z2 were considered very acidic and acidic, respectively. In Z3, the rhizospheric pH varied between 6.01 ± 0.15 in *O. natrix* and 7.20 ± 0.01 in *H. scoparium*. Concerning conductivity, in general no remarkable differences were observed between plant species sampled from ZC and Z2, except in rhizospheric soil of *A. canariense* from Z2, which presented high values of conductivity compared to other species (0.58 ± 0.04 mS cm^{-1}). In Z3, conductivity was slightly higher if compared to ZC and Z2, with values varying between 0.29 ± 0.01 and 0.31 ± 0.02 mS cm^{-1}. However, the highest conductivity values were observed in rhizospheric soils from Z1, ranging from 1.24 ± 0.03 to 1.31 ± 0.08 mS cm^{-1}, reflecting a high salinity in this location. The levels of TOC, OM, and extractable P in rhizospheric soils decreased with increasing metal contamination, being in average 6.6, 6, and 3.7 times lower, respectively, in the three contaminated zones than in ZC. TOC and OM contents were significantly higher in ZC followed by Z3, while in Z1 and Z2, no significative differences were observed. These parameters were quite similar between the different plant species sampled from the same location, with exception of *P. harmala* rhizospheric soil in Z2, where TOC ($1.21 \pm 0.151\%$) and OM ($2.08 \pm 0.10b\%$) values were significantly higher. A similar trend was observed for TN, and the maximum values were determined in the rhizospheric soils sampled from ZC (0.09–0.40%), followed by Z3 (0.13–0.19%), Z2 (0.05–0.18%), and Z1 (0.04–0.12%). Extractable P was significantly higher in the rhizospheric soils of ZC (0.12 ± 0.005–0.61 ± 0.060 mg kg^1) followed by the Z3 (0.13 ± 0.015–0.20 ± 0.007 mg kg^1).

Total metal concentrations varied between plant species and zones. Cu was the main contaminant present in the rhizosphere of all plant species and mine tailings, followed by Zn and Pb. Overall, metal concentrations were higher in mine tailings than in rhizospheric soils. As expected, the rhizospheric soils from Z1 showed the highest levels of metals, followed by Z2, while soils from Z3 showed the lower contamination. In fact, the concentration of total Cu was in average 19, 16, and 6 times higher in soils from Z1, Z2, and Z3, respectively, than in soils collected from ZC. A similar trend was observed for Pb; however, for Zn and Cr, the tendency was different with rhizospheric soils from ZC showing higher levels of both metals. Some differences were also observed between plant species in each sampling zone. In general, the rhizosphere of *P. harmala* presented higher levels of metals, especially Cu, when compared to the other plant species in all sampling zones where this plant was found. The highest Cu- and Zn-extractable concentrations were recorded in the rhizosphere of metallophytes from Z1, while in the other sampling zones, the levels were very similar.

3.2 Germination test

The germination rates of common wheat and lettuce seeds growing in mine tailings and in the different rhizospheric soils are presented in Fig. 2. In comparison to wheat, lettuce seeds were more sensitive to the phytotoxic effects of metals and to the acid pH. Complete inhibition of seed germination of both species was observed in mine tailings, contrary to what happened in ZC, where seeds germinated perfectly. Seed germination rates were lower in rhizospheric soils from Z1 than in soils collected from moderate (Z2) and low (Z3) metal-contaminated zones. This tendency was particularly evident for lettuce seeds. Germination rates of lettuce seeds were similar in both Z1 rhizospheric soils, while higher germination rates were observed in the rhizospheric soils of *P. harmala* and *H. scoparium* from Z2 compared to *O. natrix* and *A. canariense*. Moreover, the germination rates in the rhizospheric soils of *O. natrix* and *H. scoparium* from Z3 were higher if compared to the soils of the same plant species in Z2.

Table 1 Physicochemical properties of the rhizospheric soils of metallophytes (*Ononis natrix*, *Haloxylon scoparium*, *Peganum harmala*, and *Aizoon canariense*) growing in the different sampling zones (ZC, control; Z1, high metal contamination; Z2, moderate metal contamination; Z3, low metal contamination) and of mine tailings (MT)

Sampling zones	Plant species	pH	EC (mS cm^{-1})	TOC (%)	OM (%)	TN (%)	C/N	P Olsen (mg g^{-1})
ZC	*O. natrix*	7.85 ± 0.05a	0.10 ± 0.02 g	2.01 ± 0.122a	3.46 ± 0.07a	0.18	11.17	0.22 ± 0.003b
	H. scoparium	8.02 ± 0.02a	0.23 ± 0.02f	1.93 ± 0.093a	3.32 ± 0.15a	0.40	4.83	0.17 ± 0.017c
	P. harmala	8.17 ± 0.03a	0.20 ± 0.04f	2.06 ± 0.105a	3.54 ± 0.18a	0.21	9.81	0.12 ± 0.005d
	A. canariense	7.38 ± 0.02b	0.21 ± 0.00f	1.98 ± 0.085a	3.41 ± 0.09a	0.09	22.00	0.61 ± 0.060a
Z1	*H. scoparium*	4.49 ± 0.03f	1.31 ± 0.08b	0.18 ± 0.050d	0.31 ± 0.09d	0.12	1.50	0.04 ± 0.000f
	P. harmala	4.92 ± 0.04e	1.24 ± 0.03c	0.12 ± 0.044d	0.21 ± 0.07d	0.04	3.00	0.06 ± 0.006f
Z2	*O. natrix*	5.82 ± 0.02d	0.11 ± 0.00 g	0.14 ± 0.026d	0.24 ± 0.04d	0.10	1.40	0.07 ± 0.009e,f
	H. scoparium	5.25 ± 0.13e	0.19 ± 0.01f	0.29 ± 0.056d	0.50 ± 0.09d	0.15	1.93	0.07 ± 0.003e,f
	P. harmala	6.73 ± 0.05c	0.22 ± 0.01f	1.21 ± 0.151b	2.08 ± 0.10b	0.18	6.72	0.04 ± 0.001f
	A. canariense	5.32 ± 0.04e	0.58 ± 0.04d	0.24 ± 0.036d	0.41 ± 0.03d	0.05	4.80	0.11 ± 0.007d,e
Z3	*O. natrix*	6.01 ± 0.15d	0.29 ± 0.01e	0.74 ± 0.044c	1.27 ± 0.06c	0.13	5.69	0.13 ± 0.015d
	H. scoparium	7.20 ± 0.01b	0.31 ± 0.02e	0.76 ± 0.064c	1.31 ± 0.10c	0.19	4.00	0.20 ± 0.007b,c
MT	Absence of plants	2.80 ± 0.02 g	2.45 ± 0.04a	0.19 ± 0.032d	0.33 ± 0.05d	0.04	4.75	0.05 ± 0.001f

Means (= 3) in same column with different letters are significantly different from each other (< 0.05) according to the Student-Newman-Keuls test

EC, electrical conductivity; *TOC*, total organic carbon; *OM*, organic matter; *TN*, total nitrogen; *C/N*, carbon-nitrogen ratio; *P Olsen* extractable phosphorus

3.3 Soil microbiology parameters

3.3.1 Dehydrogenase activity

Figure 3 shows the results of the DHA activity in mine tailings and in rhizospheric soils. The activity of DHA was completely undetectable in tailings. Soils in ZC showed high DHA activity, ranging from 23 to 47 μg TPF g^{-1} of dry soil 24 h^{-1}, with the highest values being recorded in *H. scoparium* and *P. harmala* rhizospheres. In general, DHA activity decreased with increasing metal contamination with the lowest activity being observed in the rhizosphere of *H. scoparium* and *P. harmala* in Z1 and of *O. natrix* and *A. canariense* in Z2, which did not exceed 1.9 μg TPF g^{-1} of dry soil 24 h^{-1}. In the low metal-contaminated zone (Z3), the DHA activity recorded in the rhizosphere of *H. scoparium* was similar to the values found in control soils.

3.3.2 Quantification of culturable soil microorganisms

The microbial abundance in mine tailings and in rhizospheric soils is shown in Fig. 4. As expected, no neutrophilic culturable microorganisms were found in mine tailings. Overall, the numbers of culturable microorganisms were much higher in soils from ZC, particularly in the rhizospheres of *H. scoparium* and *P. harmala*, than in metal contaminated soils. In control soils, the numbers of bacteria varied from 2.30 × 10^6 to 8.13 × 10^6 CFU g^{-1} dry soil, while actinomycetes and fungi ranged from 1.22 × 10^4 to 9.92 × 10^4 and from 1.73 × 10^3 to 6.20 × 10^3 CFU g^{-1} dry soil, respectively.

Overall, no significant differences were observed between rhizospheric soils from metal-contaminated areas with exception of the rhizosphere of *H. scoparium* in the low-contaminated zone (Z3) that showed higher microbial density when compared to the size of microbial populations found in the rhizospheric soils from moderate (Z2) and high (Z1) metal-contaminated areas.

3.3.3 Bacterial community structure and diversity in the rhizosphere of metallophytes

The DGGE band profiles obtained from the rhizosphere of metallophytes growing along the gradient of metal contamination, as well as the species richness (S) and the Shannon-Weaver (H) index, are shown in Fig. 5. The total number of band positions detected in the gel was 37 (data not shown). The species richness was generally different in each sample, varying between 7 (Z3_H) and 29 (Z1_H). In general, samples from Z1 (most contaminated area) showed in average higher species richness (25) when compared to ZC (16), Z2 (15), and Z3 (13) samples. A similar trend was observed for H' which also varied according to the metal contamination in sampling zones. The rhizosphere of *H. scoparium* collected in Z3 showed the lowest bacterial diversity (H' = 0.842), while the highest diversity was found in the rhizosphere of the same plant species collected in Z1 (H' = 1.462). Indeed, when

Table 2 Total and CaCl$_2$-extractable metal concentrations in the rhizospheric soils of metallophytes (*Ononis natrix*, *Haloxylon scoparium*, *Peganum harmala*, and *Aizoon canariense*) growing in the different sampling zones (ZC, control; Z1, high metal contamination; Z2, moderate metal contamination; Z3, low metal contamination) and of mine tailings (MT)

Sampling zones	Plant species	Total metals (mg kg^{-1})				CaCl$_2$ extractable metals (mg kg^{-1})			
		Cu	Zn	Pb	Cr	Cu	Zn	Pb	Cr
ZC	*O. natrix*	46.90	178.20	19.68	23.87	0.10	0.11	< ld	< ld
	H. scoparium	63.03	224.53	17.77	20.44	0.13	0.07	< ld	< ld
	P. harmala	66.49	263.80	25.44	20.03	0.10	0.09	< ld	< ld
	A. canariense	58.84	212.00	32.23	21.08	0.13	0.23	< ld	< ld
Z1	*H. scoparium*	1079.93	309.25	80.50	10.15	0.28	0.53	< ld	< ld
	P. harmala	1234.43	137.62	141.25	8.99	0.28	0.49	< ld	< ld
Z2	*O. natrix*	1076.98	155.93	73.07	15.93	0.22	0.06	< ld	< ld
	H. scoparium	690.45	135.58	40.53	9.59	0.15	0.11	< ld	< ld
	P. harmala	1049.89	200.33	112.80	6.11	0.09	0.10	< ld	< ld
	A. canariense	928.28	182.95	129.18	14.05	0.18	0.30	< ld	< ld
Z3	*O. natrix*	625.73	90.25	65.13	20.13	0.13	0.30	< ld	< ld
	H. scoparium	87.25	77.28	73.99	21.52	0.11	0.09	< ld	< ld
MT	Absence of plants	1657.54	290.31	103.07	37.52	24.26	3.20	< ld	< ld

comparing the bacterial diversity in rhizospheric soils of metallophytes species common to the different zones, it was observed that diversity increased with increasing metal contamination in the rhizosphere of *H. scoparium* and *P. harmala* plants. The diversity of bacterial communities of *H. scoparium* (present in the four sampling zones) was greater in the most contaminated zones (Z1 and Z2) than in less contaminated ones (ZC and Z3). Similarly, for *P. harmala*, the diversity index was higher in Z1 (H' = 1.301) and Z2 (H' = 1.176) than in ZC (1.145). Conversely, in the rhizosphere of *A. canarensis*, it was observed a decrease in bacterial diversity from ZC (H' =

1.342) to Z2 (H' = 1.175), while in the rhizospheric soils of *O. natrix*, no remarkable differences were recorded.

The cluster analysis also indicated differences in the composition of rhizospheric bacterial communities among metallophytes (Fig. 5), which seems to be mainly related to sampling zones. Overall, bacterial communities clustered into two main groups with a similarity of around 40% (Fig. 5). One of the groups enclosed only 2 samples corresponding to the rhizospheres of *O. natrix* and *H. scoparium* from ZC and Z3, respectively, while the other included the remining samples. The latter presented 2 sub-clusters, the first one clustered essentially rhizospheric soils collected in the most contaminated

Fig. 2 Effect of mine tailings (MT) and different rhizospheric soils on the germination of wheat and lettuce seeds. ZC, control; Z1, high metal contamination; Z2, moderate metal contamination; Z3, low metal contamination; MT, mine tailing). Results are expressed as mean ± SD (*n* = 3). Bars with different letters (lowercase, wheat; uppercase, lettuce) differ significantly from each other (*P* < 0.05)
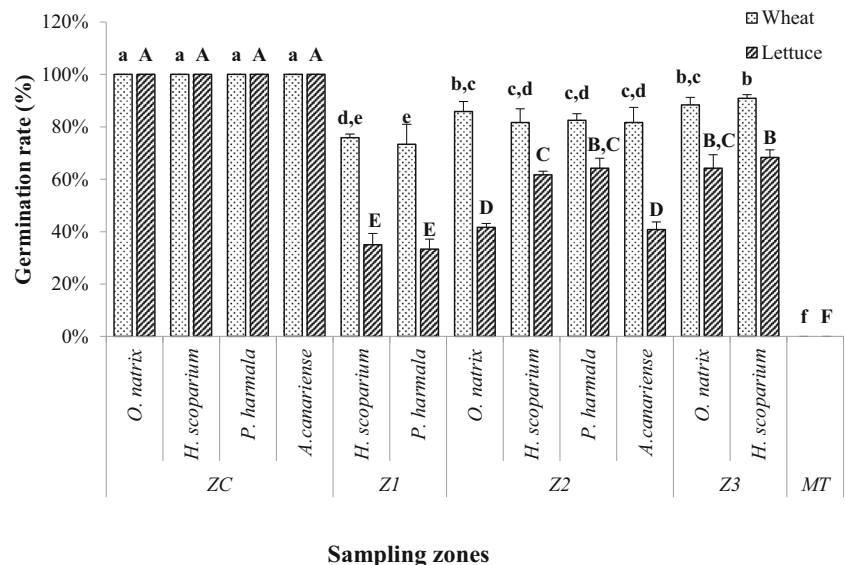
Fig. 3 Dehydrogenase activity (DHA) in mine tailing (MT) and in the rhizosphere of the different metallophytes (*O. natrix*, *H. scoparium*, *P. harmala*, *A. canariense*) collected in each sampling zone (ZC, Z1, Z2, Z3). Results are expressed as mean ± SD (*n* = 3). Bars with different letters differ significantly from each other (*P* < 0.05)
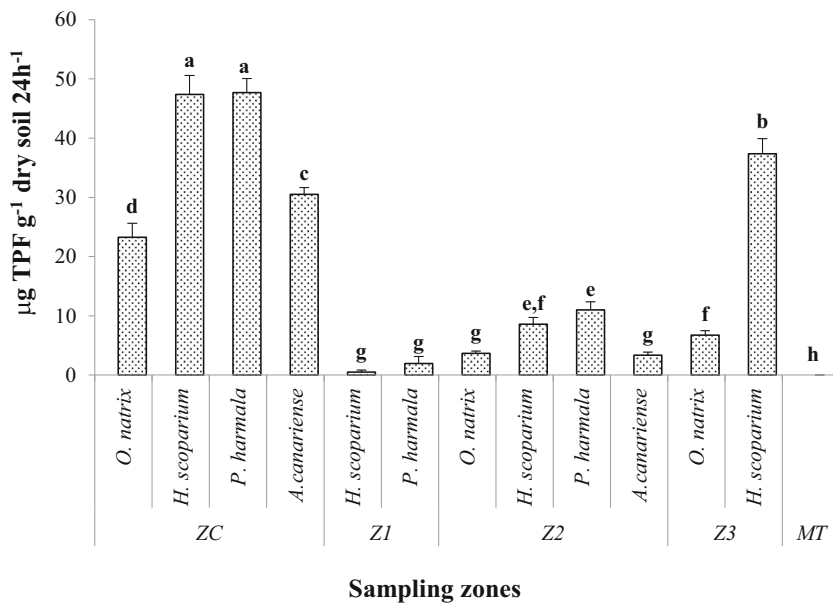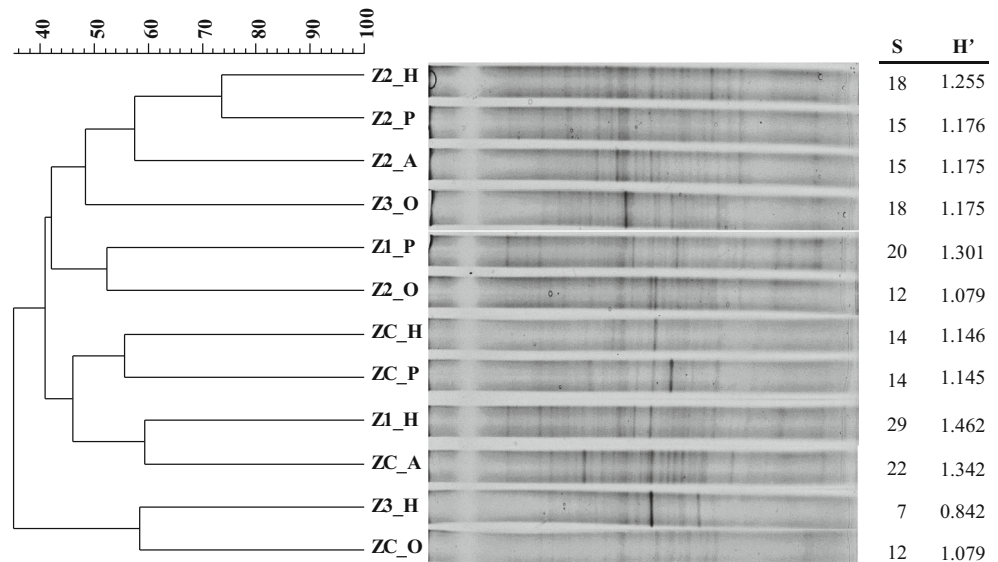
Fig. 4 Number of culturable heterotrophic bacteria (A), actinomycetes (B), and fungi (C) in mine tailing (MT) and in the rhizosphere of the different metallophytes (*O. natrix*, *H. scoparium*, *P. harmala*, *A. canariense*) collected in each sampling zone (ZC, Z1, Z2, Z3). Results are expressed as mean ± SD (*n* = 3). Bars with different letters differ significantly from each other (*P* < 0.05)
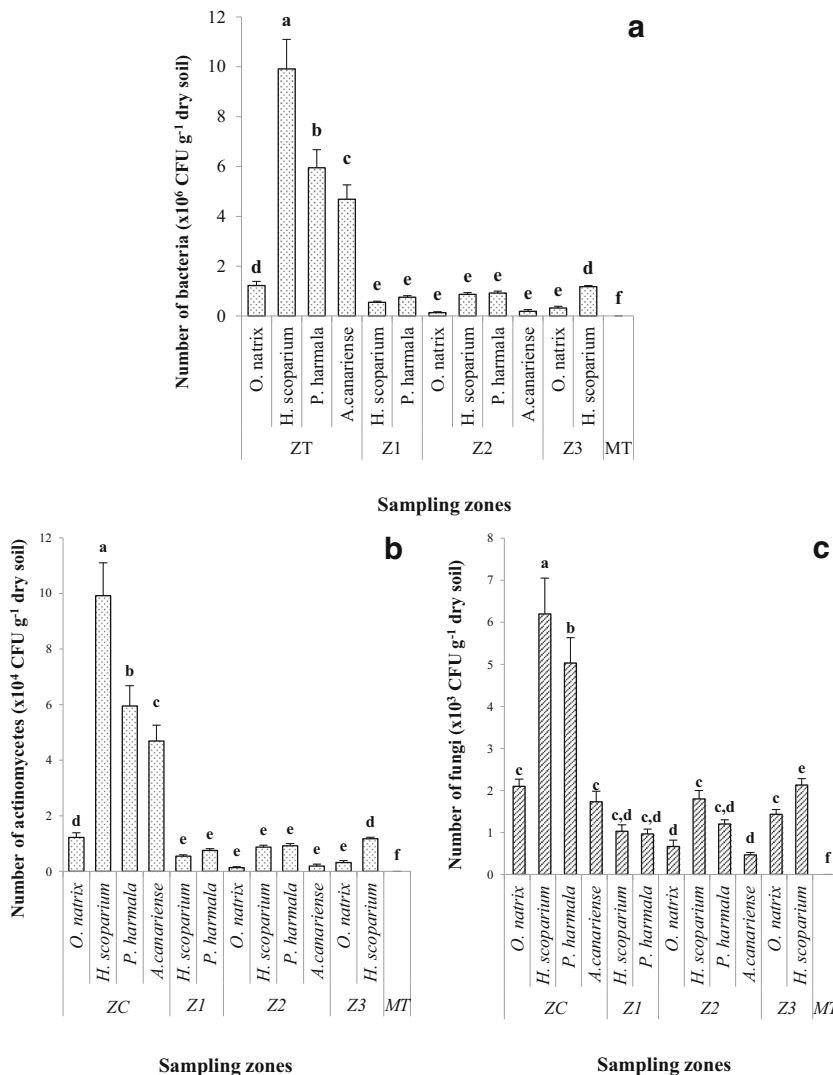
Fig. 5 DGGE profiles, cluster analysis based on UPGMA method, and Shannon index of rhizospheric soils from different metallophytes (*O. natrix*, *H. scoparium*, *P. harmala*, *A. canariense*) collected in each sampling zone (ZC, Z1, Z2, Z3)

Sample	S	H'
Z2_H	18	1.255
Z2_P	15	1.176
Z2_A	15	1.175
Z3_O	18	1.175
Z1_P	20	1.301
Z2_O	12	1.079
ZC_H	14	1.146
ZC_P	14	1.145
Z1_H	29	1.462
ZC_A	22	1.342
Z3_H	7	0.842
ZC_O	12	1.079

areas (Z1 and Z2), whereas the second comprised mostly soils retrieved from ZC. The highest percentage of similarity (74%) was found between the rhizospheres of *H. scoparium* and *P. harmala* both collected in Z2. Statistical analysis (PERMANOVA) of the DGGE data confirmed that the composition of rhizospheric bacterial communities was significantly ($P < 0.05$) influenced by sampling zone (Table S1 – Electronic Supplementary Material). The environmental variables that influenced the distribution of rhizospheric samples were represented as vectors in NMDS (Fig. 6). The total concentrations of Zn, Cr, and Pb, as well as TOC and OM contents in soils, were the variables that most impacted the composition of bacterial communities, as proved by its high correlation coefficients (> 0.5). The composition of rhizospheric bacterial communities in the same metallophyte sampled from different zones was quite different and no particular trend was observed. In accordance, PERMANOVA analysis showed that plant species and the interaction between both factors (Zone:Plant) did not significantly ($P < 0.05$) influence communities' composition.

3.4 Plant analysis

The concentrations of Cu and Zn in the shoots of metallophytes collected from the metal-contaminated zones (Z1, Z2, and Z3) were generally higher than those measured in the plants collected from ZC (Table 3). In all contaminated zones, the highest concentrations of Cu were detected in shoots of *A. canariense* and *O. natrix*, while Zn was mainly found in shoots of *O. natrix* and *P. harmala*.

The BCFs for Cu and Zn were lower than 1 in all sampling zones and plants (Table 3). For Cu, BCFs were slightly higher in control soils (ZC) than in metal-contaminated ones, while

an opposite trend was observed for Zn. The higher Zn BCFs were observed for *O. natrix* plants in Z2 and Z3.

3.5 Pearson's correlations

Pearson correlation coefficients between the different physicochemical properties and microbiological parameters of rhizospheric soils collected from Kettara mine region are shown in Table 4. The microbial abundance was positively influenced by pH, TOC, and TN contents, while the total concentrations of Cu and Pb negatively affected the numbers of all groups of microorganisms. As expected, a similar trend was observed for DHA activity, with a positive correlation of this parameter to the same physicochemical properties that influenced microbial abundance and a negative correlation to total concentrations of Cu and Pb and to extractable forms of Cu and Zn. The correlations between α-diversity and soil microbiological and physicochemical properties are shown in Table 5. Species richness was positively correlated to EC ($r = 0.662$, $P < 0.05$) and extractables fractions of Zn and Cu ($r = 0.781$, $P < 0.01$ and $r = 0.691$, $P < 0.05$, respectively), whereas H index was significantly correlated to EC ($r = 0.581^*$), total Zn ($r = 0.578$, $P < 0.05$), and Cu extractable concentrations in soils ($r = 0.691^*$).

4 Discussion

The indigenous metallophytes growing in the vicinity of Kettara mine can withstand high concentrations of metals and are well adapted to the semiarid conditions of this region. Most of them were either annual or perennial species (Midhat et al. 2016). The dominance of this kind of vegetation is due to the typical climate of arid and semi-arid zones, which is

Fig. 6 Non-metric multi-dimensional scaling (NMDS) based on DGGE data showing samples colored by zone (Z). Vectors represent the correlation (Pearson's) between the environmental variables (EC, pH, TOC, TN, OM, P Olsen, Zn, Cu, Cr, Pb, ext Cu, ext Zn) and the ordination coordinates. EC, electrical conductivity; TOC, total organic carbon; TN, total nitrogen; OM, organic matter; P Olsen, P assimilable; ext, extractable metal content. O, *O. natrix*; H, *H. scoparium*; P, *P. harmala*; A, *A. canariense*
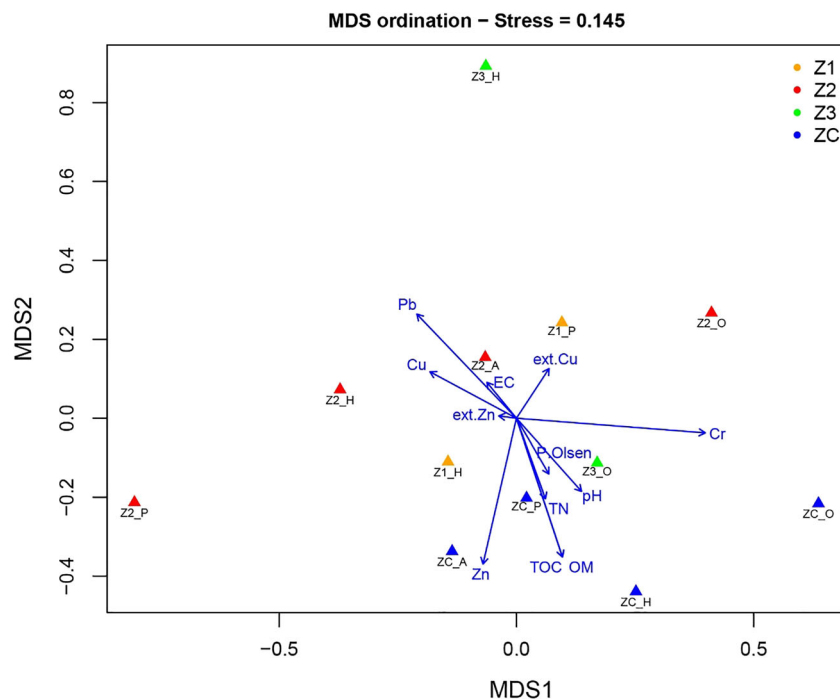
characterized by low annual precipitation. In the present work, among the four studied metallophyte species, only two (*H. scoparium* and *P. harmala*) were able to grow in the highly metal-contaminated zone (Z1, 1079.93–1234.43 mg Cu kg^{-1}, 80.50–141.5 mg Pb kg^{-1}, and 137.62–309.25 mg Zn kg^{-1}). In fact, some of these plants showed visible signs of toxicity in the field, including a slow growth when compared to the same plant species in ZC, suggesting that the bioavailable metal concentrations were high enough to induce toxicity. These

Table 3 Cu and Zn concentrations in shoots and bioconcentration factors (BCF) of metallophytes (*Ononis natrix*, *Haloxylon scoparium*, *Peganum harmala*, and *Aizoon canariense*) growing in the different sampling zones (ZC, control; Z1, high metal contamination; Z2, moderate metal contamination; Z3, low metal contamination)

Sampling zones	Plant species	Cu (mg kg^{-1})	Zn (mg kg^{-1})	BCF	
				Cu	Zn
ZC	*O. natrix*	15.92	16.33	0.34	0.09
	H. scoparium	9.83	18.93	0.16	0.08
	P. harmala	9.60	19.80	0.14	0.08
	A. canariense	17.04	27.40	0.29	0.13
Z1	*H. scoparium*	25.95	32.51	0.02	0.11
	P. harmala	14.60	27.06	0.01	0.20
Z2	*O. natrix*	53.90	46.89	0.05	0.30
	H. scoparium	21.16	33.87	0.03	0.25
	P. harmala	11.73	39.32	0.01	0.20
	A. canariense	33.50	26.28	0.04	0.14
Z3	*O. natrix*	42.51	25.86	0.07	0.29
	H. scoparium	6.31	11.87	0.07	0.15

findings are in agreement with the results of germination tests which clearly showed that high levels of total and extractable metals in rhizospheric soils collected from Z1 and Z2 impaired the germination of lettuce and wheat seeds.

The metallic concentrations found in the shoots of metallophytes of Kettara mine were in the same range of concentrations observed by other authors in native plants of mining sites (Bennisse et al. 2004; Boularbah et al. 2006b; Yoon et al. 2006; Ha et al. 2011). The concentrations of Cu in shoots of *O. natrix* and of *A. canariense* exceeded the phytotoxic concentration thresholds of 20 mg kg^{-1} proposed by Kabata-Pendias and Pendias (2001), corroborating the results previously obtained by Boularbah et al. (2006b). However, the accumulation of this metal in the aboveground tissues was very low when compared to the accumulation capacity of other metallophytes, like *Elsholtzia splendens* growing in Cu-contaminated soils (Wang et al. 2008), making their use unfeasible for phytoextraction purposes. According to Baker and Brooks (1989), the four plants sampled in the different zones of Kettara mine are not hyperaccumulators, as their BCF values were lower than 1 (Table 3), being classified as hypertolerant metallophytes (Boularbah et al. 2006b). As a result, all studied plants seem to be good candidates for improving metal stabilization and vegetation cover of mining sites, reducing the risk of off-site contamination.

The pH of soils sampled in Kettara mine was not a limiting factor for plant growth, except in mine tailings where no vegetation was found, probably as a consequence of the extremely acidic pH (2.8). A total inhibition of seed germination was also observed in mine tailing samples, which is in line with

Table 4 The Pearson correlation coefficients between soil microbiological parameters and soil physicochemical properties

	DHA	CFU^B	CFU^F	CFU^A	pH	EC	TOC	TN	P Olsen	Cu$_{tot}$	Zn$_{tot}$	Pb$_{tot}$	Cr$_{tot}$	Cu$_{ex}$	Zn$_{ex}$
DHA	1														
CFU^B	0.85**	1													
CFU^F	0.88**	0.89**	1												
CFU^A	0.83**	0.82**	0.92**	1											
pH	0.90**	0.83**	0.69*	0.73**	1										
EC	− 0.48	− 0.38	− 0.28	− 0.33	− 0.82*	1									
TOC	0.81**	0.84**	0.73**	0.70*	0.86**	− 0.52	1								
TN	0.74**	0.70*	0.77**	0.87**	0.70**	− 0.50	0.63*	1							
P Olsen	0.44	0.23	0.37	0.12	0.49	− 0.34	0.59*	0.05	1						
Cu$_{tot}$	− 0.89**	− 0.75**	− 0.65*	− 0.69*	− 0.91**	0.72**	− 0.64*	− 0.64*	− 0.62*	1					
Zn$_{tot}$	0.17	0.42	0.41	0.31	− 0.21	0.49	0.03	0.03	− 0.06	0.22	1				
Pb$_{tot}$	− 0.68*	− 0.70*	− 0.61*	− 0.67*	− 0.66*	0.53	− 0.65*	− 0.65*	− 0.47	0.79**	− 0.08	1			
Cr$_{tot}$	0.66*	0.59*	0.43	0.46	− 0.07	0.38	0	0	0.26	− 0.11	0.22	− 0.29	1		
Cu$_{ex}$	− 0.64*	− 0.49	− 0.35	− 0.45	− 0.64*	0.82**	− 0.33	− 0.33	− 0.19	0.54	0.43	0.24	0.72**	1	
Zn$_{ex}$	− 0.58*	− 0.45	− 0.35	− 0.46	− 0.72**	0.9**	− 0.42	− 0.42	− 0.21	0.61*	0.45	0.33	0.66*	0.98**	1

EC, electrical conductivity; *TOC*, total organic carbon; *TN*, total nitrogen; *P Olsen*, P assimilable; *-tot*, total metal content; *-ex*, extractable metal content; *DHA*, dehydrogenase activity; *CFU*, number of culturable cells of bacteria (B), fungi (F), and actinomycetes (A)

Significant at * $P < 0.05$; ** $P < 0.01$

what was observed in the field, confirming the crucial role of pH and metal pollution on plant development. Indeed, highly acidic pH is one of the main factors involved in the inhibition of the natural colonization of plants in mining wastes (Anawar et al. 2013; Courtney and Pietrzykowski 2018). Consequently, in the course of time, the open cast tailings become more vulnerable to runoff and wind erosion leading to a huge metal contamination of the surrounding areas (Mian and Yanful 2003; Boularbah et al. 2006a; El Khalil et al. 2008; El Hamiani et al. 2010). In this work, the pH varied from slight acidic to slight alkaline in the different sampling zones, except in Z1, where rhizospheric soils are very acidic. This may be explained by the proximity of Z1 to the mining site and consequently to a higher exposure to sulfur-rich dust. These kinds of materials suffer biological and chemical oxidation leading to soil acidification and consequent inhibition of the development of dense vegetation, which may explain the absence of some plant species, including *O. natrix* and *A. canariense*, in this site.

According to the Canadian Soil Quality Guidelines, total Cu (87.25 to 1234.43 mg Cu kg^{-1}) concentrations in rhizospheric soils of contaminated soils were in most cases above the limit recommended for agricultural, parkland, commercial, and industrial land (63–91 mg Cu kg^{-1}), use, while total Pb concentrations (40.53 to 141.25 mg Pb kg^{-1}) were above the limit recommended for agricultural and parkland (70–140 mg Pb kg^{-1}) use. In addition, according to the Dutch Standards, the levels of Cu, Pb and Zn in soils were, in general, above the target values (36 mg Cu kg^{-1}, 85 mg Pb kg^{-1}, and 140 mg Zn kg^{-1}).

Previous studies have shown that soil microbial abundance and diversity are highly dependent on environmental conditions, including soil properties (e.g., pH, EC, metals) (Yuangen et al. 2006; Khan et al. 2010), vegetation cover (Berg and Smalla 2009; Hu et al. 2010), and seasonal changes (Bamborough and Cummings 2009; Yao et al. 2011; Zhang et al. 2018). In the present work, total concentrations of Cu and Pb had a significative and negative effect on microbiological parameters as shown by the correlation factors obtained between metal concentrations and: DHA activity (− 0.89, $P < 0.01$ and − 0.68, $P < 0.01$), bacterial ($r = − 0.75$, $P < 0.01$ and − 0.70, $P < 0.05$), fungi ($r = − 0.65$, $P < 0.01$ and − 0.61, $P < 0.05$), and actinomycetes ($r = − 0.69$, $P < 0.05$ and − 0.67, $P < 0.05$) counts. DHA activity decreased with increasing metal concentrations confirming the negative effects of these pollutants on soil microbial activity. Similar results were obtained by Xie et al. (2009) that reported a decrease in DHA activity in soils contaminated with 100 mg Cu kg^{-1}. DHA activity is usually more affected by metal pollution than other soil enzymes. In fact, several studies reported dehydrogenases as very sensitive enzymes to metal pollution being frequently used as biological indicators in metal-affected soils (Fernández-Calviño et al. 2010; Hu et al. 2014; Xiao et al. 2017).

The low bacterial counts registered in most contaminated soils could be influenced by the choice of growth medium, since TSA favors the growth of heterotrophic in detriment of oligotrophic bacteria. Nonetheless, DHA was strongly correlated to microbial counts ($r > 0.8$), confirming that soil with low pH and high concentrations of metals have probably a low number of total microorganisms in their soils.

Table 5 The Pearson correlation analysis testing the relationship between α-diversity (species richness and Shannon index) and soil microbiological and physicochemical properties

	Species richness (S)	Shannon index (H)
DHA	− 0.443	− 0.427
CFU[B]	− 0.286	− 0.200
CFU[F]	− 0.261	− 0.205
CFU[A]	− 0.091	− 0.010
pH	− 0.558	− 0.507
EC	0.662*	0.581*
OC	− 0.236	− 0.165
OM	− 0.235	− 0.165
TN	− 0.352	− 0.330
P Olsen	0.053	0.044
Cu_{tot}	0.400	0.417
Zn_{tot}	0.520	0.578*
Pb_{tot}	0.143	0.132
Cr_{tot}	− 0.440	− 0.496
Cu_{ex}	0.588*	0.549
Zn_{ex}	0.781**	0.691*

EC, electrical conductivity; *OC*, organic carbon; *OM*, organic matter; *TN*, total nitrogen; *P Olsen*, P assimilable; *-tot*, total metal content; *-ex*, extractable metal content; *DHA*, dehydrogenases activity; *CFU*, number of culturable cells of bacteria (B), fungi (F), and actinomycetes (A)

Significant at * $P < 0.05$; ** $P < 0.01$

As described previously, the microbial abundance in rhizospheric soils was affected by metal pollution. However, it has been reported that pH, OM content, and soil nutrient status were also important factors influencing microorganisms in metal-contaminated sites, since they can often help to offset the harmful effects of metallic pollutants (Stefanowicz et al. 2012). In the current work, Pearson's correlations showed that TOC and TN contents were beneficial for the development of microorganisms in the rhizospheric soils. Beside to its contribution to improve soil nutrient status (e.g., C and N), the increase of OM contents will contribute to increase soil porosity, improving air circulation and water retention capacity, stimulating the growth of microorganisms (Lal 2006; Park et al. 2011). In addition, high levels of OM can promote the immobilization of trace elements in stable organic complexes with humic substances (Spark et al. 1997; Farrell et al. 2010), reducing its bioavailability (Giller et al. 2009), and creating a more suitable environment for the establishment of microorganisms and vegetation.

In our study, very low densities of actinomycetes and fungi were detected in the different rhizospheric soils, suggesting that these soils are preferred niches for bacterial populations. This could be related to the less favorable conditions in soils, including the levels of metals that cannot be tolerated by these groups of microorganisms (Deneux-Mustin et al. 2003). Khan and Scullion (2000) have also noted higher bacterial rates than

fungi in metal-contaminated soils, suggesting that fungi are more affected by metallic compounds. This could be explained by the fact that fungi generally colonize large pores and soil macro-aggregates, which makes them more exposed and vulnerable to the metal toxicity than bacteria, which are often associated to smaller pores and soil micro-aggregates (Carrasco et al. 2010).

Several studies have shown that metal contamination negatively affects the abundance, composition, and structure of bacterial communities, which generally results in a decreased diversity (Khan et al. 2007; Wang et al. 2008). The areas in the vicinity of the old Cu mine have been subject to heavy metal pollution over a long period of time, which may explain the reduction of the microbial abundance and the depletion of the DHA activity in contaminated zones (Z1, Z2, and Z3). A different trend was observed by Pereira et al. (2015) that reported higher bacterial numbers in the rhizosphere of *Juncus effuses* and *Phragmites australis* growing in a heavily metal-polluted soil than in the less contaminated one. Nevertheless, the analysis of DGGE profiles did not show a negative effect of metal pollution on bacterial diversity; quite in the contrary, in comparison to ZC, the highest bacterial diversity was recorded in the rhizospheric soils retrieved from the most metal-contaminated area (Z1), suggesting that long-term metal exposure led to the development of well adapted and diversified bacterial populations. These findings were corroborated by the positive and significant correlations obtained between both diversity indexes and total Zn concentrations and Zn and Cu extractable fractions in soils. In accordance, Martínez-Iñigo et al. (2009) also reported new bands in DGGE profiles of the rhizospheric communities of *Silene vulgaris* in response to metal exposure. Xiao et al. (2017) also found that the diversity of microbial communities increased after the application of high levels of vanadium in the soil, suggesting that new species of metal tolerant bacteria have replicated under these conditions.

The composition and diversity of bacterial communities may also be strongly influenced by the quantity and quality of root exudates (Marschner et al. 2004), which in turn is dependent on the type and concentration of metals (Qin et al. 2007; Luo et al. 2017). This could explain, in part, the differences observed in the bacterial diversity of rhizospheric soils of *H. scoparium* among sampling sites, which varied from very low in Z3 to very high in Z1. However, it is important to note that the lower diversity registered in Z3 was accompanied by a large increase of bacterial numbers.

In the present work, the composition and diversity of rhizobacterial communities were more influenced by sampling zones and the associated environmental factors than by plant species, which is in agreement with the results obtained by Lopez et al. (2017) that showed that bacterial diversity in the rhizosphere of *Alyssum mural* was more conditioned by the physicochemical factors than by plant species.

Conversely, Pacwa-Płociniczak et al. (2018) showed that plants have a higher impact on soil microbial community than metallic pollutants.

5 Conclusions

The indigenous metallophytes growing in the vicinity of Kettara mine are not metal hyperaccumulators, making them good candidates for phytostabilization purposes, in particular *H. scoparium* and *P. harmala*. Both species provided an improvement of microbial abundance and bacterial diversity in their rhizospheres if compared to other metallophytes, especially under metal contamination, evidencing their ability to be used in the revegetation of mining areas, reducing the risk of dispersion of metals through erosion and leaching.

Microbial abundance and DHA activity are good tools to evaluate the effect of anthropogenic disturbances on soil microbial functions, since both parameters were systematically impacted by increasing metal concentrations in soils, while the influence of plant species was poorly noticed.

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