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Diversity of Soil Microbial Communities from an Iron Mining Area (Oued Zem, Morocco)

Pestrost mikrobioloških združb v tleh rudarskega območja Oued Zem, Maroko

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Abstract

Microbial communities in the soil were collected from 20 samples of an iron mining area (Ait Ammar, Oued Zem, Morocco), and unaffected samples were analysed to identify the effects of metal concentrations on functional diversity (Biolog® EcoPlates), and structural diversity (polymerase chain reaction–denaturing gradient gel electrophoresis of 16S rDNA). *Aliivibrio fischeri* is mainly used for evaluating polluted soil. The functional diversity was assessed by using such indices as area under substrate utilisation curve, richness, Shannon–Weaver and evenness indices. The analysis of similarities and the non-metric multidimensional scaling analyses of DGGE profiles showed that metals in the soil do not have a significant influence on bacteria. Principal component analysis of Biolog data revealed the similarity in the metabolic profiles of mining samples. These results suggest that the direction and the distance from the iron mine tailings do not have significant effects on the metabolic and structural diversity of the soil bacterial population. The toxicity of metals in soils heavily contaminated with Fe and P did not affect the quantities of microbial populations and did not significantly change the microbial diversity of contaminated soils.

Key words: Biolog® EcoPlates; PCR-DGGE; Microtox®; microbial communities; iron mine

Izvleček

Mikrobne združbe smo vzorčili v dvajsetih vzorcih tal z območja rudnika železa Ait Ammar, Oued Zem. V neporušenih vzorcih smo ugotavljali vplive vsebnosti kovin na funkcionalno pestrost mikrobnih združb z Biolog® EcoPlates in strukturno pestrost z PCR-DGGE (16 S rDNA). Bakterijo *Aliivibrio fischeri* pogosto uporabljajo kot bioindikator onesnaženosti tal. Funkcionalno pestrost smo ocenili z uporabo indikatorjev kot so območje pod krivuljo izraženosti barve substrata, Richness, Shannnon-Weaver in Evenness indeks. Analize mere podobnosti in nemetrično večrazsežnostno lestvičenje DGGE profilov so pokazale, da kovine v tleh nimajo značilnih vplivov na bakterijo. Statistična analiza glavnih komponent podatkov Biolog je razkrila podobnost metabolnih profilov v orudenih vzorcih. Rezultati navajajo, da smer in oddaljenost lokacije vzorčenja od jalovišč rudnika železove rude nima značilnega vpliva na metabolno in strukturno pestrost bakterij v tleh. Toksičnost kovin v tleh močno onesnaženih z Fe in P ni vplivala na številčnost mikrobnih združb in ni značilno spremenila mikrobiološke pestrosti v onesnaženih tleh.

Ključne besede: Biolog® EcoPlates; PCR-DGGE; Microtox®; mikrobiološke združbe, rudnik železa

Introduction

The properties of microbial populations have been drawing international consideration, particularly in relation to the environmental aspects. Various studies have focussed on microbial community behaviour both under different types of land use and in contaminated mining areas [1].

In what concerns metal contamination, bacteria of soil can avoid toxicity to themselves by changing metals into nontoxic forms [2], immobilising metals on the cell surface [3] or in intracellular polymers, developing metal efflux pumps, volatilising metals by enzymatic reactions, forming extracellular capsules that hold the metal [4], siderophore–metal interaction and precipitation, or biomethylation [5].

Previous approaches of analysing bacterial environments were principally concentrated on determining biomass, estimating respiration or analysing cultivable and/or uncultivable bacteria [6]. These methods are not appropriate for the examination of microbial population but provide information concerning isolation procedures or estimation of amounts of microorganisms. Furthermore, most of these methods necessitate the usage of growth medium on which just 1.0–10% of soil microbes can develop [7].

Biotechnological methods have been currently used to replicate the topsoil environment and these have supported the investigation of bacterial diversity [8]. Knowledge of microbial diversity has increased with the ability to survey genes directly in environmental samples. The 16S ribosomal RNA (*rRNA*) gene has been the target of the majority of such ecological molecular surveys because of its usefulness as a prokaryotic phylogenetic marker [9]. The 16S rDNA is suitable for these analyses because this gene exists in all prokaryotic microorganisms and is hence useful in bacterial identification.

Denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified 16S rDNA amplicons obtained from total DNA, which was described by Muyzer et al. (1993) [10], as a new culture-independent genetic fingerprinting technique, is a faster method for describing microbial community structure and diversity in ecological samples [11]. The most important benefit of this method is that it permits the precise resolution of microbial diversity, making it superior to cloning and subsequent sequencing. However, this does not exclude the use of the latter techniques for identifying the key microorganisms [12].

In this study, 20 soil samples, gathered from an abandoned iron mine located in the suburbs of Oued Zem City in the centre of Morocco, as well as reference samples, were collected [13], to study the soil bacterial populations in the samples. The microbial communities were characterised using the Biolog® system and the DGGE of the 16s rDNA gene parts that were amplified from the total bacterial DNA extracted from the soil. The major objectives of this work were to obtain information about the microbial diversity and the potential toxicity of the soils that were polluted by mixture of metals and phosphorus from the iron mine.

Materials and methods

Sample collection and total community DNA isolation

Soil samples were collected from a heavy metal-contaminated site and a non-polluted control soil as described by Nouri et al. [13]. The contamination reflects 78 years of exposure history [14]. Samples from the polluted and reference areas were taken from the top 20 cm. Samples were congealed at –20°C until analysis. DNA was obtained directly from soils and purified with the Ultra Clean™ Soil DNA Isolation Kit® (MoBio Laboratories, Inc., Carlsbad, CA, USA) as per the manufacturer guidelines. The quality of extracted DNA was checked on 1% agarose gels stained with RedSafe™ DNA Nucleic Acid Staining Solution (20000×). The extracted DNA was stored at –20°C for future applications.

PCR amplification of 16S rRNA gene

In this study, a nested PCR method was utilised [15]. The primers used in this study are recorded in Table 1. Total bacterial DNA extracted from soil was amplified in the initial PCR. The universal primers F27 [16] and R1512 [17] were utilised to amplify 1450 bp of the 16S *rRNA* gene fragment following the protocol established by Nogueira et al. [18], with a C1000™ Thermal Cy-

Table 1 *Primers used in first and nested PCR*

PCR	Primer	Sequence (5′-3′)
First PCR	F27	AGAGTTTGATC(A/C)TGGCTCAG
	R1512	ACGGTTACCTTGTTACGACTT
Nested PCR	F984	AACGCGAAGAACCTTAC
	R1378	CGGTGTGTACAAGGCCCGGGAACG
	GC-clamp	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG 5′ attached to F984GC

cler (Bio-Rad, Hercules, CA, USA). The amplified products were separated on 1% (g/ml) agarose gels containing RedSafe™ DNA Nucleic Acid Staining Solution (20000x) and visualised under ultraviolet light. Negative and positive controls were incorporated in all PCR experiments. The PCR products obtained from the primer pair F27-R1512 were used for a double PCR with the universal primer pair F984-GC and R1378 for amplification of bacterial 16S rDNA portions (473 bp) [19], according to Nogueira et al. (2012) [18]. The amplified bands were visualised in the same manner as the first PCR.

Denaturing gradient gel electrophoresis

The DGGE of the amplified bacterial amplicons was conducted utilising the D-code apparatus (Universal Mutation Detection System, Bio-Rad, Hercules, CA, USA). Amplified bacterial 16S rDNA portions were separated in an 8% polyacrylamide gel with a denaturing gradient of 32%–72%. The electrophoresis was run in 1× TAE (Tris–acetate–EDTA) buffer at 60°C at a voltage of 75 V for 16 h and was conducted after a pre-run at 220 V for 10 min. The DGGE gels were silver-stained according to Heuer et al. (1997) [19]. After staining, the DGGE gels were digitalised and analysed. The gel was photographed with Molecular Imager FX apparatus (Bio-Rad, Hercules, CA, USA).

Community catabolic profiles

Microbial functional diversity was evaluated by using the Biolog® EcoPlate system (Biolog Inc., Hayward, CA, USA). Approximately 2 g was weighed into two test tubes, which were developed in the dark at 20–28°C for one week. Later, 20 ml of a 0.2% pyrophosphate solution was added to each tube. After mixing, 1 ml of the sample was transferred into a new tube, and 18 ml pyrophosphate solution was added. Then, 140 ml aliquots of this solution were put in EcoPlate wells. The plates were developed in the dark at 20–28°C for 15 days, and the absorbance of the plates was recorded to times a day with a microplate reader at 590 nm, ending when the wells no longer changed colour.

Absorbance values found with EcoPlates were corrected with the colour intensity at time 0; each substrate value was then corrected to double that of the control well. By utilising the values, the average well colour development (AWCD) was determined. All negative values were set to zero. AWCD was calculated as the mean of the corrected absorbance values for all wells, which were utilised to determine the area under the absorbance curve (AUC). For each observation, the values of the corrected optical density at 590 nm (OD_{590}) were utilised to calculate the metabolic diversity of soil microbial populations by determining the number of substrates utilised ($OD_{590} > 0.15$) by the community (richness) [20]. To evaluate the effect of contamination, substrate use data were divided into six substrate classes indicating various substrate groups (amines, amino acids, carbohydrates, carboxylic acids, polymers and miscellaneous) and the average absorbance in each class was tested.

Microtox® test

The Microtox® test, based on the reduction of the bioluminescence of the *Aliivibrio fischeri,* is utilised as a sign of contamination in the sample. Calculating the half-maximal effective concentration (EC_{50}) revealed information about the soil toxicity. The Microtox® was implemented on solid phase, according to the procedures for the basic solid phase test (BSPT), as per the guideline process [21]. Light emission was recorded after 30 min of contact and the production data were studied with MicrotoxOmni

software Version 1.18. Concentration necessitated the provocation of toxic effect on 50% of the community (EC_{50}) (percentage dry weight/volume), which was revealed for each soil sample. Furthermore, toxicity data were classified utilising a French classification founded on the EC_{50} values [22]. After assessment of the quality of soils, four toxicity scores (0–3) were determined: insignificant (EC_{50} >10, score 0), low (0.5< EC_{50} <10, score 1), moderate (0.2< EC_{50} <0.5, score 2) and high (EC_{50} <0.2, score 3).

Data analysis

The DGGE gels were analysed as described by Nogueira et al. (2012) [18]. For community-level physiological profiling (CLPP), each Ecoplate™ was provided in triplicates, which were utilised to determine the AWCD. Kinetics of microbial activity was evaluated through the AUC vs. time profile. The AUC values were determined from plate readings after 15 days of incubation and used for advanced analyses. PCA was utilised for the analysis of CLPP. Richness (S), Shannon–Wiener (H) and Evenness (E_H) indices were determined according to the following equations:

$$H = -\sum_{i=1}^{N} Pi(lnPi) \quad (1)$$

$$EH = H/Hmax = H/lnS \quad (2)$$

where Pi is the ratio of the activity on a special substrate relative to the sum of activities on all substrates and S is the substrate richness [23]. The AUC values and the diversity indices of the contaminated soil samples were compared with those of the control soil. Significantly higher responses between contaminated and non-contaminated samples were determined by the Student's *t*-test.

Multifactor analysis of variance (2-way ANOVA) was used to test the differences between each mine site and the reference for each substrate class. The Tukey's honestly significant differences (HSD) test for multiple comparisons was run if significant dissimilarities were obtained ($p < 0.05$). The PCA was used to analyse the Biolog® data.

The similarities between samples were found by non-metric multidimensional scaling (MDS).

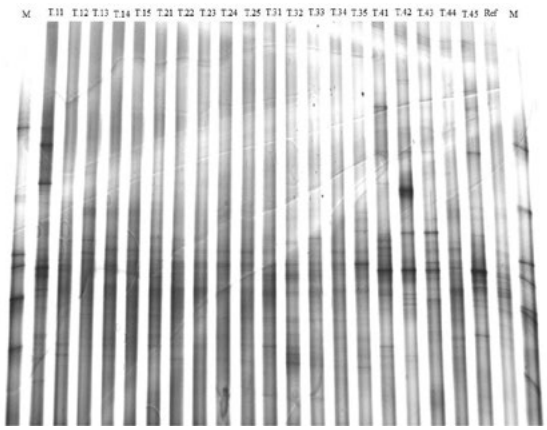

Figure 1: *DGGE profiles of the PCR products found from the 16S rDNA separated from total DNA soil obtained from 4 transects (T.1–T.4) with 5 points each (1–5) and the reference soil. M corresponds to the bacterial marker.*

Consequently, an analysis of similarities (ANOSIM) was calculated to define the significant dissimilarities, both between samplings (AUC) or between samples' toxicity (substrate groups). Similarity percentage (SIMPER) analysis was used to check the substrates that contributed most towards the differences between the different sites. The similarity matrix was found with Bray–Curtis similarity of the AUC values converted as log(x+1). The 2-Way ANOVA and Student's *t*-test analyses were performed with SPSS software (version 17.0); the MDS, PCA and the ANOSIM were evaluated with PRIMER 5 software.

Results

PCR-DGGE for bacterial community structure

The physicochemical parameters and the contents of metals and phosphorus present in the studied soil taken from the mining area have been previously reported by Nouri et al. (2014) [13]. PCR-DGGE was utilised to investigate the variations in the structure of the bacterial community in 20 samples at different distances from the abandoned iron mine. DGGE profiles obtained from the worldwide bacterial primers (F984 and R1378) show the structural profiles of soil samples (Figure 1).

ANOSIM results revealed that no significant difference existed between transects (global

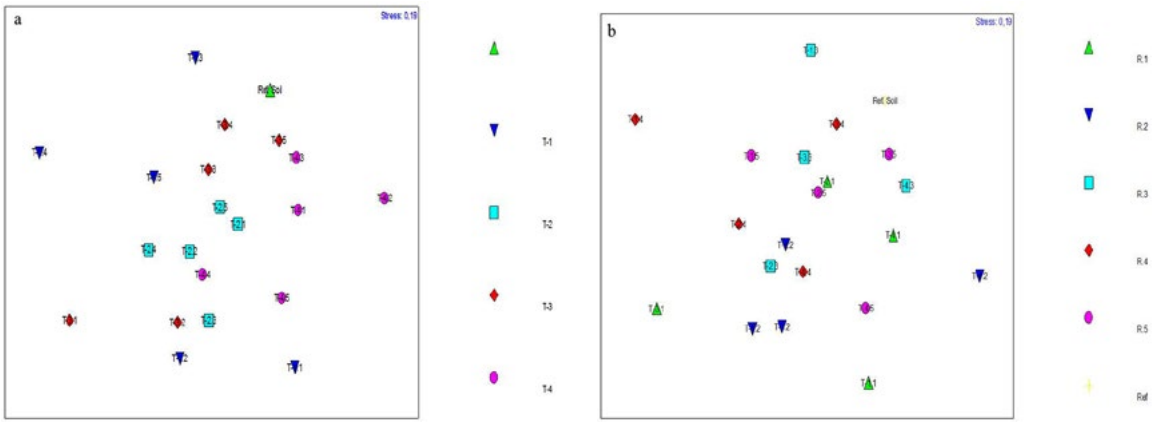

Figure 2: *Non metric multidimensional scaling analysis of the DGGE profiles obtained from total community DNA of soil samples collected along 4 transects (T.1–T.4) with 5 points each (1–5) and a reference soil. Analysis was made a) by transect and b) by round.*

R = 0.162, p = 0.049) and that there are no significant differences among the samples obtained from different directions. The results show again that no significant difference (global R = –0.027, p = 0.625) was found between samples from different rounds, so the distance of the mine does not significantly influence the bacteria in the soil. Consequently, the total metal contamination of the soil has no significant influence on the microbial population.

Non-metric MDS analyses corroborate the ANOSIM results, indicating that samples from the mine area are clearly segregated (Figure 2). The plot of the MDS analysis of the DGGE fingerprints does not reveal a clear correlation between the soil samples (Figure 2a, b). The stress level applied in this study for MDS evaluation was 0.19 (Figure 2), indicating that the two-dimensional representation is valid. In fact, our results suggest that the distance (round 2b) and direction (transect 2a) had less effect on the microbial community in Ait Ammar abandoned iron mine.

Shannon-Wiener index (H′) was used to estimate the diversity of bacterial populations in each sample. Table 2 shows that the diversity is high.

Table 2: *Shannon-Wiener index (H') of bacteria in each sample.*

Samples	H′
T.11	4,310
T.12	3,8434,319
T.13	
T.14	3,525
T.15	4,178
T.21	4,420
T.22	4,228
T.23	3,835
T.24	4,317
T.25	4,557
T.31	4,445
T.32	4,191
T.33	4,566
T.34	4,247
T.35	4,339
T.41	4,439
T.42	3,975
T.43	4,477
T.44	4,398
T.45	4,196
Réf.	4,317

Biolog® analysis

The data revealed were examined in two ways. First, the rate of change of colour intensity on the plates over time was revealed by evaluating the AUC for each substrate, and then the profiles for different places were compared by PCA.

Comparison of the average AUC for the AWCD and for each substrate of the sampling sites in the mine area and the reference soil is shown in Table 3. Results shows that the responses were lower for all substrates in mine soils compared to the reference soil, except α-cyclodextrin. Diversity of substrate utilisation determined by the substrate richness (S), Shannon–Wiener's index (H′) and Evenness index (E_H) for different soil samples was also not significantly different between mine soils and reference ones. So no significant differences were found between reference and soils of the iron mines (Table 3). Table 3 shows that the functional groups (amines,

Table 3: *Diversity indices of substrate use and analysis of soil samples in Ecoplate substrates after 266 h of incubation.*

Substrates	*t*-test	2-way ANOVA
Amines		
Phenylethylamine	-	A
Putrescine	-	
Amino acids		
Glycyl-L-glutamic Acid	-	
L-Arginine	-	*
L-Asparagine	-	(T.11-3), (T.15),
L-Phenylalanine	-	(T.23-5), (T.31),
L-Serine	-	(T.34-5), (T.43-4).
L-Threonine	-	
Carbohydrates		
β-Methyl-D-glucoside	-	
D-Xylose	-	
i-Erythritol	-	*
D- Mannitol	-	(T.15).
N-Acetyl-D-glucosamine	-	
D-Cellobiose	-	
α-D-Lactose	-	
Carboxylic Acids		
D- Galactonic acid γ-lactone	-	
D-Galacturonic acid	-	
2-Hydroxy benzoic acid	-	*
4-Hydroxy benzoic acid	-	(T.11-3), (T.15),
γ-Hydroxybutyric acid	-	(T.23-5), (T.31-5),
D-Glucosaminic acid	-	(T.43).
Itaconic acid	-	
α-Ketobutyric acid	-	
D-Malic acid	-	
Miscellaneous		
Pyruvic acid methyl ester	-	
Glucose-1-phosphate	-	A
D,L-α-Glycerol phosphate	-	
Polymers		
Tween 40	-	A
Tween 80	-	
α-Cyclodextrin	+	
Glycogen	-	
AWCD	-	nd
H	ns	nd
S	ns	nd
E_H	ns	nd

AUC values and diversity indices of soil samples were compared to those of the reference soil. Differences were found by the Student's t-test (+, sample value greater than reference; –, sample value lower than reference).

**The mean difference is significant at the 0.05 level, except the sites indicated between parentheses.*

[a]*The mean difference is not significant at the 0.05 level. ns, the difference is not significant; nd, not determined.*

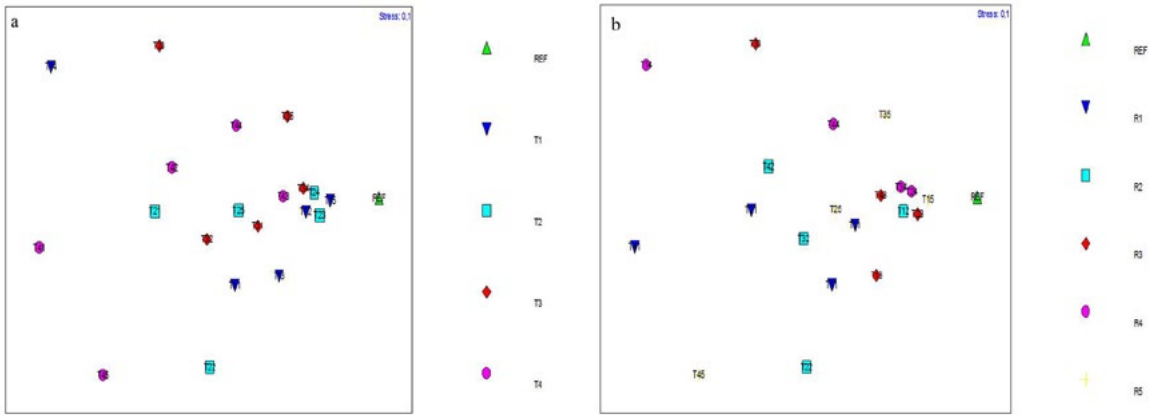

Figure 3: *Non-metric multidimensional scaling analysis of average AUC for eco-substrates obtained from 4 transects (T.1–T.4) with 5 points each (1–5) and a reference soil. Analysis made a) by transect; b) by round.*

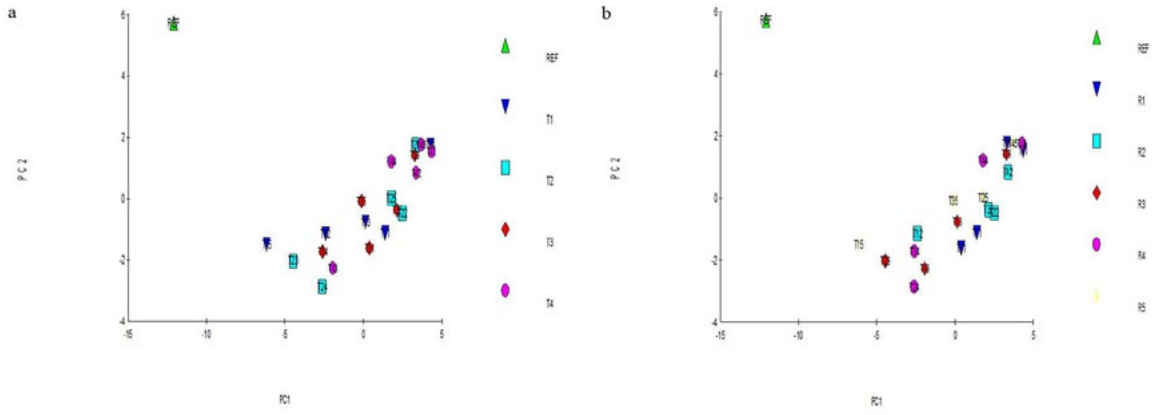

Figure 4: *Plot of principal component analysis (PCA) of substrate utilisation by soil microbial communities a) by transect; b) by round.*

miscellaneous and polymers) did not differ between the mining and the reference samples. For the amino acids and carboxylic acids, eight mining site samples were significantly different from the reference, but twelve site samples were not different (site samples between parentheses). However, for carbohydrates, all site samples were different from the reference, except T.1.5, which was not different.

MDS analysis performed on 31 carbon sources of EcoPlates showed that total metal contamination did not noticeably affect the microbial community profiles, considering potential carbon utilisation (Figure 3). The same result was determined using ANOSIM analysis: global R = 0.022, p = 0.334 between transects and reference; and global R = –0.036, p = 0.644 between rounds and reference. The low SIMPER dissimilarity illustrated the meaning of these results by displaying the group dissimilarity and the most contributive substrate. The greatest average dissimilarity between reference and rounds was 25.25 (REF, R1) and between reference and transects was 25.15 (REF, T4). The most contributive substrate for the first and second dissimilarities was itaconic acid (7.15%, 5.69% respectively). For further determination of microbial communities in different places, Biolog data after 266.5 h were subjected to PCA (data not shown); results showed that there was only one component accounting for 97.02% of the variation, included all soil samples and is strongly correlated, indicating no changes in the functional activities of soil microbial populations inhabiting the soil with distance at the Ait Ammar abandoned iron mine.

PCA using all 31 substrates did not reveal a partition of the samples of mining soil, presenting

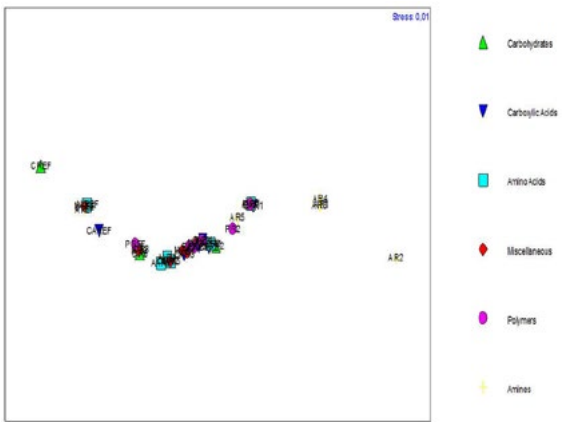

Figure 5: *Non-metric multidimensional scaling of substrate group utilisation by microbial samples obtained from different mine distances.*

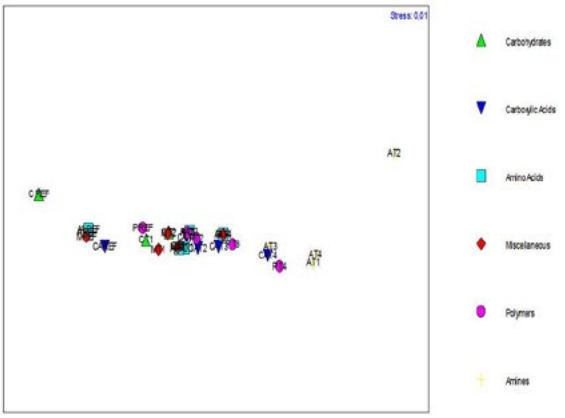

Figure 6: *Non-metric multidimensional scaling of group utilisation in samples from different mine directions.*

similar patterns of potential carbon use and comparable microbial communities. The PCA graphs (Figure 4) revealed similarity in the metabolic profiles of contaminated samples.
The MDS executed at the substrate group level, on the samples from different mine distances (Figure 5) and mine directions (Figure 6), revealed that the group use pattern varies essentially for amines. A difference was detected in the carbohydrates used by the different bacterial communities between the mine and no reference sites.

Microtox® toxicity assay

Ecotoxicity assessment can provide refined information on the changes of soil quality. In this study, Microtox® assay was performed to study toxicity levels of the soil, and the results are shown in Table 4. Toxicity results are expressed as EC_{50}. The EC_{50} is a commonly used endpoint for the comparative assessment of the toxicity results. The determination of the EC_{50} threshold constitutes one of the most commonly used values for ecotoxicity tests. Based on the EC_{50} values, the toxicity level was insignificant or not toxic for all soil samples, except sample T.4.3, which showed low toxicity level.

Discussion

The goal of this present work was to study the effects of total metal concentrations on microbial genetic and functional diversity and on the bioluminescence of the bacterium *A. fischeri* in abandoned iron mine soil, using 16S rDNA gene profiles produced by PCR-DGGE, functional analyses conducted with the Biolog EcoPlate and toxicity tests generated by Microtox. The soil samples contained heavy metals, iron, and phosphorus [13]. Therefore, the soil environment presented conditions that appear undesirable to microbial populations and needed to be examined.
The bioluminescence test has been used in this study because it is favoured over several bacterial testing methods; and it is among the most appropriate tests for soil and sediment toxicity evaluation [24]. BSPT Microtox® test has been utilised to assess the toxicity of polluted soils or sediments [25]. The Microtox test showed that, in most cases, the reduction of the luminescence was not significant (Table 4). De Castro-Català et al. (2016) [26] reported high ecotoxicity due to bioavailability of metals. The metal bioavailability in the Ait Ammar research area was very low [13]. Thus, the smaller toxic effects of the soil samples could partly be explained by the lesser metal bioavailability.
The grouping of the PCR-DGGE and Biolog approaches was found to be beneficial in systematically understanding microbial populations in mining soil. The reasons for using a combined approach were that soil functionality was thought to be dependent on not only the microbial species present but also on the potential metabolic activity of the microorganisms in this soil. So, for better comprehension of the

Table 4: *Changes in soil microbial community Microtox® toxicity influenced by contaminated and uncontaminated soils.*

Soil	EC_{50} (%)	Toxicity score	Toxicity level
T.11	6.99	0	in[b]
T.12	nt[a]	-	-
T.13	2.13	0	in
T.14	nt	-	-
T.15	nt	-	-
T.21	713.90	0	in
T.22	18.21	0	in
T.23	nt	-	-
T.24	nt	-	-
T.25	20.37	0	in
T.31	nt	-	-
T.32	1.14	0	in
T.33	9.65	0	in
T.34	nt	-	-
T.35	nt	-	-
T.41	nt	-	-
T.42	nt	-	-
T.43	0.66	1	Low
T.44	nt	-	-
T.45	128.80	0	in
REF	nt	-	-

[a] *nt: not toxic*

[b] *in: insignificant*

effect of total metal contamination on microbial populations in iron mine soil, we applied the PCR-DGGE and Biolog methods to evaluate the metabolic activity and structural diversity. These two methods were used by many researchers [27–29].

The microbial metabolic activity in soil samples was described by AUC of the substrates set up on the Biolog EcoPlate. ANOSIM, PCA and MDS analyses of Biolog results revealed that the CLPP from mining and control samples are not significantly different. Moreover, the microbial diversity index and multivariate comparisons indicated that no significant differences were found between mining and reference soils. So, total metal contamination did not considerably affect the microbial communities inhabiting Ait Ammar iron mine soil. These findings could be explained with the very low metal bioavailability and soil properties. Girvan et al. (2003) [30] concluded that the soil parameters had a significant impact on soil microbial populations, of which soil pH was supposed to exert the major influence on the constitution of soil bacterial populations [31]. Ma et al. (2016) [32] reported that metal accessibility is low when the pH is about 6.5–7. Among the soil samples tested herein, 85% were neutral and 10% were slightly acidic [13]. On the other hand, no significant difference in the soil pH between mining and reference samples was revealed. In addition, iron and phosphorus reduce the available fraction of metals in soil and thus minimise the risks associated with their mobility (e.g., ecotoxicity) [33–36].

In addition, we obtained a negative correlation between bioavailable Cd, Cu, Zn and Fe concentrations and all measures of species richness, Shannon's diversity as well as evenness indices (data not shown). Moreover, we obtained a significant negative correlation between bioavailable Cd concentration and evenness index. Remarkably, Berg et al. (2012) [37] discovered that long-term contact with Cu affects the bacterial population structure, but not its diversity and species richness, arguing that various metals influence the populations differently. Similar observations were made by Chodak et al. (2013) [38]. Other metals and phosphorus did not correlate with the diversity variables, which could have been produced by the low available concentrations (Cr and Pb) or the low toxicity (P) [13].

For entirely understanding the soil bacterial population structure, additional investigation approaches should be necessitated. DGGE is a useful method for examining genetic modification in soil microbial structure. In this study, consistent with Biolog results, the MDS and ANOSIM results suggest that the distance and direction had less effect on the microbial community. So, total metal contamination of soil had no significant influence on the microbial population. Furthermore, the diversity of bacterial populations in each soil sample was high, but this diversity was not significantly different between mining soils compared with reference ones (Table 2). However, our results were in contrast with previous studies conducted by Bååth (1989) [39], Hu et al. (2007) [40] and Zhu et al. (2013) [41], which showed that heavy metal pollution reduced the biomass and diversity of the soil bacterial community. Moreover, our results were consistent with those found by

Fritze et al. (2000) [42]. These authors concluded that no reduction of the microbial biomass is revealed in the presence of heavy metals. Moreover, recent studies have revealed that after a century of exposure to acid drainage, the bacterial community tends to stabilise and increase its diversity, resulting in an environment with a microbial diversity similar to that of non-impacted areas [43, 44]. The Ait Ammar mine was in operation for 27 years and was abandoned for 51 years, and soil contamination is considered to be relatively ancient. Furthermore, it has been suggested that while acute exposure has a negative influence on bacterial diversity, in chronically contaminated environments, the proliferation of resistant bacteria can increase diversity [45, 46]. This case is common in mines such as the Ait Ammar iron mine.

In this study, no change in structural diversity was discovered. Comparable results were obtained utilising the Biolog and Microtox tests. This, however, may not be surprising because over the past years, Fe oxides were obtained from the Center for the Progress of New Remediation Technologies due to their sorptive and reactive nature [47] and utilised for the elimination of Cr, Pb, Cu, Cr and Cd [47].

Mamindy-Pajany et al. (2013) [22] concluded that hematite and zero-valent iron permitted decreasing the leakage of metals by 50% and reduced the toxicity of contaminated marine sediments. Those "assisted natural remediation" treatments diminish heavy metal concentrations in soil and decrease microbial toxicity [48]. Furthermore, He et al. (2011) [49] reported that the addition of iron oxide magnetic nanoparticles can potentially encourage some bacterial development and modification of the soil bacterial community structure, but bacterial abundance is not modified. Therefore, numerous Fe-based remediation technologies are still at an experimental stage and require proof of their effective application in the large-scale field [48]. As well as iron-based reduction of metal solubility, phosphate amendments are also effective in reducing availability of heavy metals [33, 34]. Consequently, the normal functioning of a microbial community in the soils near the Ait Ammar abandoned iron mine might have not been weakened.

In addition, it is probable to acquire heavy metal tolerance in the ecosystem because the genes in several microorganisms directing metal resistance are located in plasmids. Moreover, in areas contaminated with heavy metals, it has been noted that the abundance of tolerant bacteria increases, while that of the more sensitive ones diminishes [50]. Therefore, the greater levels of total metals such as Cu, Cr, Zn and Fe in the soil samples can have functioned to select the resistant bacteria that dominated this ecosystem (underway results). Certain researchers have investigated the bacterial heavy metal resistance [51], which is probably due to mechanisms of tolerance and detoxification [52] as well as by the production of chelating agents [53] that attach to metals and decrease their toxicity. Furthermore, Moore et al. (2005) [54] revealed that many genes influenced by metal stress were controlled by metalloregulatory proteins. Microorganisms can also produce a multiplicity of metal-binding peptides and proteins, e.g. metallothioneins and phytochelatins, which control metal ion homeostasis and influence toxic responses [55]. Koechler et al. (2015) [56] reported that the development of a solid biofilm that is more resistant has been detected at higher concentrations of Fe^{2+}, Al^{3+}, Cu^{2+}, Fe^{3+} and Zn^{2+}. The biofilm may aid the microorganisms to develop better tolerance to toxic metals. It has been hypothesised that these structural changes may affect the metal bioavailability, cellular nutrient acquisition processes and the metabolic activities [56]. In the case of *R. alamii* YAS34, the biofilm is more solid in the presence of sub-inhibitory concentrations of cadmium than in the absence of this metal [57].

The main findings in this work were that, in the presence of high contents of Fe and P and with a neutral soil pH, the Ait Ammar soil had no acute toxicity; in addition, total metals did not affect the quantities of microbial populations and did not significantly change the metabolic diversity of mining soils. Nevertheless, other biotic and abiotic properties, not studied herein, could affect the microbial communities and can provide interesting data to help predict their ecology.

Conclusions

In this study, both functional and structural diversities of microbial communities were examined. Our results showed that the analysed soils did not present acute toxicity; Further, the MDS, PCA and ANOSIM analyses showed that there were no differences between the soil samples in terms of the structure and metabolic functions of the bacterial communities. Furthermore, the microbial diversity index and multivariate comparisons revealed that no significant differences were found between mining and reference soils. The obtained results suggest that Zn, Cr, Cu, Pb and Cd, in presence of Fe and P, did not have negative impacts on the structural and metabolic diversity of bacterial populations. Moreover, carbohydrate was mostly utilised and amines were the less functional groups used in this mining area. Enhancing our comprehension of this essential area of microbiology and exploiting it in treatments such as bioremediation and other biotechnology processes will remarkably necessitate a multidisciplinary method.

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References

[1] Wang, Q., Wang, R., Tian, C., Yu, Y., Zhang, Y., Dai, J. (2012): Using microbial community functioning as the complementary environmental condition indicator: A case study of an iron deposit tailing area. *European Journal of Soil Biology,* 51, pp. 22–29.

[2] Xu, L., Luo, M., Li, W., Wei, X., Xie, K., Liu, L., Jiang, C., Liu, H. (2011): Reduction of hexavalent chromium by *Pannonibacter phragmitetus* LSSE-09 stimulated with external electron donors under alkaline conditions. *Journal of Hazardous Materials,* 185, pp. 1169–1176.

[3] Qiu, J.H., Zhang, Y.W., Zhang, Y.T., Zhang, H.Q., Liu, J.D. (2011): Synthesis and antibacterial activity of copper-immobilized membrane comprising grafted poly (4-vinylpyridine) chains. *Journal of Colloid and Interface Science,* 354, pp. 152–159.

[4] Van Elsas, J.D., Trevors, J.T., Wellington, E.M.H. (1997): *Modern soil microbiology*. New York: Marcel Dekker; 683 p.

[5] Amico, E.D., Cavalca, L., Andreoni, V. (2005): Analysis of rhizobacterial communities in perennial Graminaceae from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria. *FEMS Microbiology Ecology,* 52, pp. 153–162.

[6] Bloem, J., Veninga, M., Sheppard, J. (1995): Fully automated determination of soil bacterium numbers, cell volumes, and frequencies of dividing cells by confocal laser scanning microscopy and image analysis. *Applied and Environmental Microbiology,* 61, pp. 926–936.

[7] Li, Z., Xu, J., Tang, C., Wu, J., Muhammad, A., Wang, H. (2006): Application of 16S rDNA-PCR amplification and DGGE fingerprinting for detection of shift in microbial community diversity in Cu-, Zn-, and Cd-contaminated paddy soils. *Chemosphere,* 62, pp. 1374–80.

[8] Borneman, J., Skroch, P.W., O'Sullivan, K.M., Palus, J.A., Rumjanek, N.G., Jansen, J.L., Nienhuis, J., Triplett, E.W. (1996): Molecular microbial diversity of an agricultural soil in Wisconsin. *Applied and Environmental Microbiology,* 62, pp. 1935–1943.

[9] Hugenholtz, P., Goebel, B.M., Pace, N.R. (1998): Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology,* 180, pp. 4765–4774.

[10] Muyzer, G., de Waal, E.C., Uitterlinden, A.G. (1993): Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology,* 59, pp. 695–700.

[11] De Oliveira, V.M., Manfio, G.P., Da Costa Coutinho, H.L., Keijzer-Wolters, A.C., Van Elsas, J.D. (2006): A ribosomal RNA gene intergenic spacer based PCR and DGGE fingerprinting method for the analysis of specific rhizobial communities in soil. *Journal of Microbiological Methods,* 64, pp. 366–379.

[12] Rombaut, G., Uantika, G.S., Boon, N., Maertens, S., Dhert, P., Top, E.M., Sorgeloos, P., Verstraete, W. (2001): Monitoring of the evolving diversity of the microbial community present in rotifer cultures. *Aquaculture,* 198, pp. 237–252.

[13] Nouri, M., Gonçalves, F., Sousa, J.P., Römbke, J., Ksibi, M., Pereira, R., Haddioui, A. (2014): Metal concentrations and metal mobility in Ait Ammar Moroccan mining site. *Journal of Materials and Environmental Science,* 5, pp. 271–280.

[14] Nouri, M., Gonçalves, F., Sousa, J.P., Römbke, J., Ksibi, M., Pereira, R., Haddioui, A. (2013): Metal Uptake by Spontaneous Vegetation in an abandoned iron mine from a Semiarid Area in Center Morocco: Implications for Phytoextraction. *Environmental Research, Engineering and Management,* 64, pp. 59–71.

[15] Gomes, N.C.M., Borges, L.R., Paranhos, R., Pinto, F.N., Mendoça-Hagler, F.N., Smalla, K. (2008): Exploring the diversity of bacterial communities in sediments of urban mangrove forests. *FEMS Microbiology Ecology,* 66, pp. 96–109.

[16] Gomes, N.C.M., Heuer, H., Schonfeld, J., Costa, R., Mendonc-a-Hagler, L., Smalla, K. (2001): Bacterial diversity of the rhizosphere of maize (Zea mays) grownin tropical soil studied by temperature gradient gel electrophoresis. *Plant and Soil,* 232, pp. 167–180.

[17] Cawthorn, D.M., Botha, S., Witthuhn, R.C. (2008): Evaluation of different methods for the detection and identification of *Enterobacter sakazakii* isolated from South African infant formula milks and the processing environment. *International Journal of Food Microbiology,* 127, pp. 129–138.

[18] Nogueira, V., Lopes, I., Rocha-Santos, T., Santos, A.L., Rasteiro, G.M., Antunes, F., Gonçalves, F., Soares, A.M.V.M., Cunha, A., Almeida, A., Gomes, N.C.M., Pereira, R. (2012): Impact of organic and inorganic nanomaterials in the soil microbial community structure. *Science of the Total Environment,* 424, pp. 344–350.

[19] Heuer, H., Krsek, M., Baker, P., Smalla, K., Wellington, E. (1997): Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environment Microbiology,* 63, pp. 3233–3241.

[20] Garau, G., Castaldi, P., Santona, L., Deiana, P., Melis, P. (2007): Influence of red mud, zeolite and lime on heavy metal immobilization, culturable heterotrophic microbial populations and enzyme activities in a contaminated soil. *Geoderma,* 142, pp. 47–57.

[21] Azur Environmental (1998): Microtox Basic Solid-phase Test (Basic SPT). CA, USA.

[22] Mamindy-Pajany, Y., Hurel, C., Geret, F., Roméo, M., Marmier, N. (2013): Comparison of mineral-based amendments for ex-situ stabilization of trace elements (As, Cd, Cu, Mo, Ni, Zn) in marine dredged sediments: A pilot-scale experiment. *Journal of Hazardous Materials,* 252–253, pp. 213–219.

[23] Zak, J.C., Willig, M.R., Moorhead, D.L., Wildman, H.G. (1994): Functional diversity of microbial communities: a quantitative approach. *Soil Biology and Biochemistry,* 26, pp. 1101–1108.

[24] Doherty, F.G. (2001): A Review of the Microtox® Toxicity Test System for Assessing the Toxicity of Sediments and Soils. *Water Quality Research Journal Of Canada,* 36, pp. 475–518.

[25] Guerra, R., Pasteris, A., Ponti, M., Fabbri, D., Bruzzi, L. (2007): Impact of dredging in a shallow coastal lagoon: Microtox® Basic Solid-Phase Test, trace metals and Corophium bioassay. *Environment International,* 33, pp. 469–473.

[26] De Castro-Català, N., Kuzmanovic, M., Roig, N., Jordi Sierra, J., Ginebreda, A., Barceló, D., Pérez, S., Petrovic, M., Picó, Y., Schuhmacher, M., Muñoz, I. (2016): Ecotoxicity of sediments in rivers: Invertebrate community, toxicity bioassays and the toxic unit approach as complementary assessment tools. *Science of the Total Environment,* 540, pp. 297–306.

[27] Xue, D., Yao, H.Y., Ge, D.Y., Huang, C.Y. (2008): Soil microbial community structure in diverse land use systems: A comparative study using Biolog, DGGE and PLFA analyses. *Pedosphere,* 18, pp. 653–663.

[28] Cycoń, M., Markowicz, A., Piotrowska-Seget, Z. (2013): Structural and functional diversity of bacterial community in soil treated with the herbicide napropamide estimated by the DGGE, CLPP and r/K-strategy approaches. *Applied Soil Ecology,* 72, pp. 242–250.

[29] Mulling, B.T.M., Soeter, A.M., Van der Geest, H.G., Admiraal, W. (2014): Changes in the planktonic microbial community during residence in a surface flow constructed wetland used for tertiary wastewater treatmen. *Science of the Total Environment,* 466–467, pp. 881–887.

[30] Girvan, M.S., Bullimore, J., Pretty, J.N., Osborn, A.M., Ball, A.S. (2003): Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soil. *Applied and environmental microbiology,* 69, pp. 1800–1809.

[31] Landesman, W.J., Nelson, D.M., Fitzpatrick, M.C. (2014): Soil properties and tree species drive b-diversity of soil bacterial communities. *Soil Biology and Biochemistry,* 76, pp. 201–209.

[32] Ma, X., Zuo, H., Tian, M., Zhang, L., Meng, J., Zhou, X., Min, N., Chang, X., Liu, L. (2016): Assessment of heavy metals contamination in sediments from three adjacent regions of the Yellow River using metal chemical fractions and multivariate analysis techniques. *Chemosphere,* 144, pp. 264–272.

[33] Cao, R.X., Ma, L.Q., Chen, M., Singh, S.P., Harris, W.G. (2003): Phosphate-induced metal immobilization in a contaminated site. *Environmental Pollution,* 122, pp. 19–28.

[34] Chen, S.B., Zhua, Y.G., Ma, Y.B. (2006): The effect of grain size of rock phosphate amendment on metal immobilization in contaminated soils. *Journal of Hazardous Materials,* 134, pp. 74–79.

[35] Komárek, M., Vaněk, A., Ettler, V. (2013): Chemical stabilization of metals and arsenic in contaminated soils using oxides. *Environmental Pollution,* 172, pp. 9–22.

[36] Garau, G., Silvetti, M., Castaldi, P., Mele, E., Deiana, P., Deiana, S. (2014): Stabilising metal(loid)s in soil with iron and aluminium-based products: Microbial, biochemical and plant growth impact. *Journal of Environmental Management,* 139, pp. 146–153.

[37] Berg, J., Brandt, K.K., Al-Soud, W.A., Holm, P.E., Hansen, L.H. Sørensen, S.J., Nybroe, O. (2012): Selection for Cu-tolerant bacterial communities with altered composition, but unaltered richness, via long-term Cu exposure. *Applied and Environmental Microbiology,* 78, pp. 7438–7446.

[38] Chodak, M., Gołębiewski, M., Morawska-Płoskonka, J., Kuduk, K., Niklińska, M. (2013): Diversity of microorganisms from forest soils differently polluted with heavy metals. *Applied Soil Ecology,* 64, pp. 7–14.

[39] Bååth, E. (1989): Effects of heavy metals in soil on microbial processes and populations. *Water, Air, & Soil Pollution,* 47, pp. 335–379.

[40] Hu, Q., Qi, H.Y., Zeng, J.H., Zhang, H.X. (2007): Bacterial diversity in soils around a lead and zinc mine. *Journal of environmental sciences,* 19, pp. 74–79.

[41] Zhu, J., Zhang, J., Li, Q., Han, T., Xie, J., Hu, Y., Chai, L. (2013): Phylogenetic analysis of bacterial community composition in sediment contaminated with multiple heavy metals from the Xiangjiang River in China. *Marine pollution bulletin,* 70, pp. 134–139.

[42] Fritze, H., Perkiömäki, J., Saarela, U., Katainen, R., Tikka, P., Yrjälä, K., Karp, M., Haimi, J., Romantschuk, M. (2000): Effect of Cd-containing wood ash on the microflora of contiferous forest humus. *FEMS Microbiology Ecology,* 32, pp. 43–51.

[43] Reis, M.P., Barbosa, F.A.R., Chartone-Souza, E., Nascimento, A.M.A. (2013): The prokaryotic community of a historically mining-impacted tropical stream sediment is as diverse as that from a pristine stream sediment. *Extremophiles,* 17, pp. 301–309.

[44] Bouskill, N., Barker-Finkel, J., Galloway, T.S., Handy, R.D., Ford, T.E. (2010): Temporal bacterial diversity associated with metal-contaminated river sediments. *Ecotoxicology,* 19, pp. 317–328.

[45] Roesch, L.F.W., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K., Kent, A.D., Daroub, S.H., Camargo, F.A., Farmerie, W.G., Triplett, E.W. (2007): Pyrosequencing enumerates and contrasts soil microbial diversity. *International Society for Microbial Ecology Journal,* 1, pp. 283–290.

[46] Sun, M.Y., Dafforn, K.A., Johnston, E.L., Brown, M.V. (2013): Core sediment bacteria drive community response to anthropogenic contamination over multiple environmental gradients. *Environmental Microbiology,* 15, pp. 2517–2531.

[47] Xu, P., Zeng, G.M., Huang, D.L., Feng, C.L., Hu, S., Zhao, M.H., Lai, C., Wei, Z., Huang, C., Xie, G.X., Liu, Z.F. (2012): Use of iron oxide nanomaterials in wastewater treatment: a review. *Science of the Total Environment,* 424, pp. 1–10.

[48] Cundy, A.B., Hopkinson, L., Whitby, R.L.D. (2008): Use of iron-based technologies in contaminated land and groundwater remediation: a review. *Science of the Total Environment,* 400, pp. 42–51.

[49] He, S., Zhang, Y., Gu, N., Feng, Y., Lin, X., Ren, H. (2011): The impact of iron oxide magnetic nanoparticles on the soil bacterial community. *Journal of Soils and Sediments,* 11, pp. 1408–1417.

[50] Kunito, T., Saeki, K., Oyaizu, H., Matsumoto, S. (1999): Influences of copper forms on the toxicity to microorganisms in soils. *Ecotoxicology and environmental safety,* 44, pp. 174–181.

[51] Silver, S., Phung, L.T. (1996): Bacterial heavy metal resistance: new surprises. *Annual review of microbiology,* 50, pp. 753–89.

[52] Gadd, G.M., Griffiths, A.J. (1977): Microorganisms and heavy metal toxicity. *Microbial ecology,* 4, pp. 303–317.

[53] Nowack, B. (2008): Chelating agents and the environment. *Environmental Pollution,* 153, pp. 1–2.

[54] Moore, C.M., Gaballa, A., Hui, M., Ye, R.W., Helmann, J.D. (2005): Genetic and physiological responses of *Bacillus subtilis* to metal ion stress. *Molecular Microbiology,* 57, pp. 27–40.

[55] Avery, S.V. (2001): Metal toxicity in yeast and the role of oxidative stress. *Advances in Applied Microbiology,* 49, pp. 111–142.

[56] Koechler, S., Farasin, J., Cleiss-Arnold, J., Arsène-Ploetze, F. (2015): Toxic metal resistance in biofilms: Diversity of microbial responses and their evolution. *Research in Microbiology,* 166, pp. 764–773.

[57] Schue, M., Fekete, A., Ortet, P., Brutesco, C., Heulin, T., Schmitt-Kopplin, P., Achouak, W., Santaella, C. (2011): Modulation of metabolism and switching to biofilm prevail over exopolysaccharide production in the response of *Rhizobium alamii* to cadmium. *PLoS ONE* 6, e26771.