

DIVERSITY AND STRUCTURE OF SOIL BACTERIAL
COMMUNITIES IN THE AREA OF NON-FERROUS METAL
PLANT REVEALED BY 16S rRNA GENE RETRIEVAL

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Abstract

The goals of the study were to assess the diversity and structure of the bacterial communities within the soil depth along a gradient of heavy metal (HM) contamination and to identify indigenous bacterial species in the agricultural area of a non-ferrous metal plant KCM 2000 Group (Plovdiv) by using 16S rRNA gene retrieval. 16S rRNA gene clone libraries were constructed for nine samples, which were collected from two soil depths in June 2020. From each library, up to 100 clones were analysed and grouped into operational taxonomic units (OTUs) by restriction fragment length polymorphism (RFLP). The representatives of the OTUs were sequenced, followed by phylogenetic analysis. The results revealed that phyla Proteobacteria (11.11–71.43%) and Actinomycetota (3.57–33%) were the most abundant. Surface soils (12 phyla and 15 classes) were more diverse than subsurface ones (7 phyla and 12 classes). The lowest diversity at both phylum and class levels was calculated for the moderately contaminated soils from the two studied depths. Thirteen 16S rDNA sequences were identified to a species level, and they belonged to Proteobacteria, Actinomycetota and Firmicutes. This study highlighted that both HM contamination and soil depth caused shifts in diversity and structure of soil bacterial communities.

Key words: soil, heavy metals, 16S rRNA gene, bacterial diversity

Introduction. The increased presence of heavy metals can disrupt the composition, community structure, and function of microbial communities, thereby affecting soil health and ecological stability [1]. Shifts in bacterial diversity and community structure under HM impact were the research focus of many authors utilizing diverse molecular approaches [2]. One of these methods, known as the “gold standard” is the 16S rRNA gene retrieval [3]. With the advances in next-generation DNA-sequencing technologies, many authors used the targeted amplicon 16S rRNA gene sequencing as a preferable method to explore microbial community structure. The aforementioned methods reveal the taxonomic composition of the microbial communities, but at different levels of details [4]. Although the classical PCR-based 16S rRNA gene surveys are less high-throughput than 16S rRNA amplicon sequencing and may not capture the full complexity of microbial communities, it allows the identification and isolation of specific bacterial species. This can be useful for further characterization, such as studying the physiology, biochemistry, or functional capabilities of bacterial communities [5].

Previous studies demonstrated that HMs have a negative impact on microbial abundance and change the structure and function of bacterial communities [3,6,7]. It has been shown that high levels of HMs might decrease the richness and diversity of soil bacterial communities, but increase the number of special bacterial taxa [8]. Besides, it has been reported that microbial richness and diversity decrease by increasing the soil depth as a common trend in various soils [7].

The goals of this study were: (i) to assess the bacterial diversity and changes in the structure of their communities throughout the soil depth along a gradient of HM contamination in agricultural lands, and (ii) to identify indigenous bacterial species, associated with increased resistance to HMs by using 16S rRNA gene retrieval.

Materials and methods. Study sites and soil sampling. The study area is located in the surroundings of a non-ferrous metal plant “KCM 2000 Group” (Plovdiv, Southern Bulgaria). Five surface (0–20 cm) and subsurface (20–40 cm) sub-samples per sampling site were collected in June 2020 as described in [6]. Sampling sites were named KCM_1 (42°03'31.68"N, 24°49'19.2"E), KCM_2 (42°03'5.76"N, 24°49'19.6"E), KCM_3 (42°02'6"N, 24°49'19.2"E), KCM_4 (42°03'31.68"N, 24°49'45.12"E), and KCM_5 (42°04'23.52"N, 24°49'45.12"E), and the surface and sub-surface soil samples were referred to as KCM_1.1, KCM_2.1, KCM_3.1, KCM_4.1, and KCM_5.1, and KCM_1.2, KCM_2.2, KCM_4.2 and KCM_5.2, respectively.

Heavy metal/metalloid concentrations. The concentrations of Zn, Pb, Cd, Cu, As were measured according to [6].

DNA extraction. Soil DNA was extracted (0.5 g) as described in [6].

PCR amplification and clone library construction. Bacterial 16S rRNA genes were amplified by PCR using the bacterial primer 8F (5'-AGAGTTT GATYMTGGCTCAG-3') and universal primer 1513R (5'-TACGGYTACCTTGT

TACGACTT-3'), and 16S rDNA clone libraries were constructed as described in [3]. The positive clones were grouped via RFLP to obtain OTUs, and the representatives of each OTU were sequenced in Macrogen Europe (the Netherlands).

Phylogenetic and data analysis. The sequences were compared to sequences available in the NCBI database (<https://www.ncbi.nlm.nih.gov>) using the BLAST tool. Then, the sequences were aligned with their closest phylogenetic relatives with ClustalW. A phylogenetic tree was constructed according to the neighbour-joining method with MEGA 11 software. The indices of richness (Chao1 and ACE) and diversity (Shannon-Weaver) were calculated with Past 4.13 software. Pearson correlation analysis was conducted to elucidate the relationship between taxa distribution and both HM concentrations and soil depth.

Nucleotide sequence accession numbers. The sequences were deposited in GenBank under the following accession numbers: OQ422575-OQ422639; OQ422707-OQ422751; OQ422861-OQ422916.

Results. Heavy metal/metalloid concentrations. The concentrations of HMs in the soils are presented in Table 1. In most of the soils, HMs exceeded the respective maximal permissible concentration (MPC) according to Bulgarian Regulation 3/2008 (<http://eea.government.bg/bg/legislation/soil>), in some cases (KCM_1.1) by 92 (Cd) to 116 (Pb) times. Soil As concentrations were under the MPC, except in KCM_1.1, KCM_1.2, KCM_4.1, and KCM_4.2. According to the HM concentrations, soils were classified as heavily (KCM_1, KCM_2 and KCM_4), moderately (KIM_5) and slightly (KCM_3.1) contaminated.

Characterization of the 16S rDNA clone libraries. Table 2 presents a summary of bacterial diversity indices. The number of OTUs ranged from 42 to 68. The PCR products of 166 clones were sequenced. The calculated indices defined KCM_4.2 and KCM_5.1 as the most bacterial diverse soils with the

T a b l e 1

Heavy metal and metalloid concentrations in soils (mg kg⁻¹), and maximum permissible concentration (MPC) according to Bulgarian Regulation 3/2008

Soils	Zn	Pb	Cd	Cu	As
KCM_1.1	9452	11569	184.9	891.4	191
KCM_2.1	1558.2	1370.1	15.7	138.9	13.4
KCM_3.1	216.2	135.6	3.9	53.7	9.6
KCM_4.1	6872	5723	86.2	570.3	80
KCM_5.1	740	335	9.3	98.2	15.5
KCM_1.2	3457.1	3744	61.8	331.1	60.6
KCM_2.2	1711.9	1480.3	38.6	155.1	5
KCM_4.2	4843.1	3424	55.7	341.5	63.5
KCM_5.2	810	330	9	88.2	12.7
MPC	320	100	2	150	25

T a b l e 2

Statistical analysis of 16S rDNA clone libraries derived from KCM soils

Soils	Number of OTUs	Number of sequences	Chao1	ACE	Shannon
KCM_1.1	42	57	77.67	93.93	3.708
KCM_2.1	54	13	258.1	288.8	4.207
KCM_3.1	60	56	162.3	202.8	4.301
KCM_4.1	46	10	119.3	200.7	3.897
KCM_5.1	68	9	1033	742.4	4.494
KCM_1.2	60	8	766.7	504	4.237
KCM_2.2	63	7	470.6	715.9	4.466
KCM_4.2	56	2	1414	1138	4.186
KCM_5.2	56	4	334	262.1	4.314

highest richness of genotypes, the opposite of KCM_1.1, where the lowest values of Chao1, ACE and Shannon indices were recorded.

Taxonomic composition of soil bacterial communities at the phylum and class levels. The phylogenetic diversity of bacteria was presented by twelve (surface soils) and seven (subsurface soils) phyla (Fig. 1A). Proteobacteria, Actinomycetota, Acidobacteriota, Bacteroidota, Firmicutes, Fibrobacterota and Thermodesulfobacteriota were distributed throughout the soil depth, whereas Gemmatimonadota, Chloroflexota, Verrucomicrobiota, Planctomycetota and Myxococcota were presented only in the surface soil layer in limited sites (KCM_2.1, KCM_3.1, KCM_4.1), probably due to some local peculiarities.

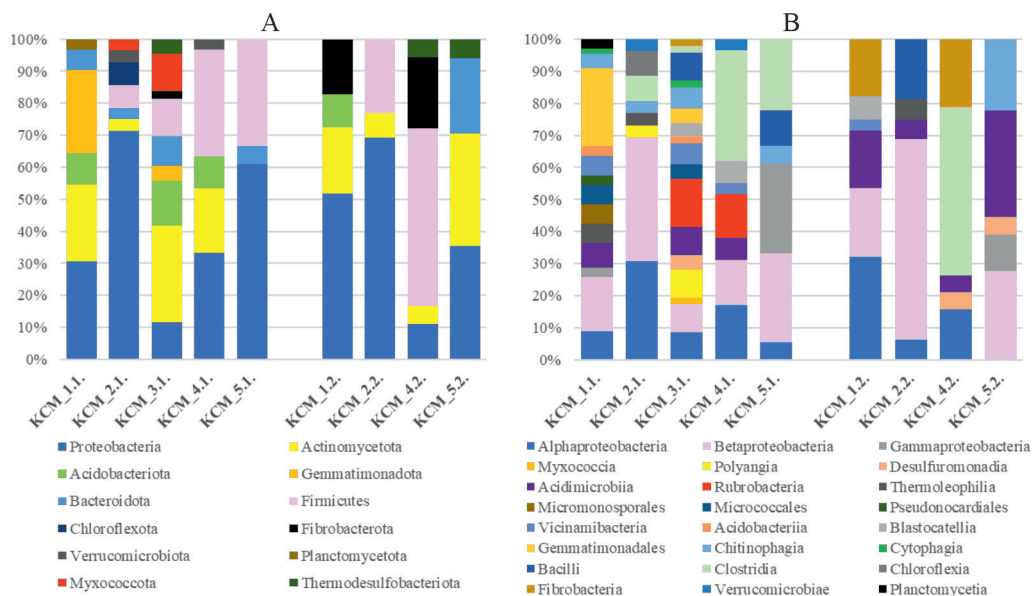


Fig. 1. Relative distribution of bacterial phyla (A) and classes (B) in KCM soils

The KCM soils were distinctly dominated by phylum Proteobacteria (30.65–71.43%), except in KCM_3.1 (11.63%) and KCM_4.2 (11.11%), where the dominants were Actinomycetota (30.23%) and Firmicutes (55.56%), respectively. Actinomycetota and Firmicutes were defined also as dominants in KCM_5.2 (Actinomycetota; 35.29%) and KCM_4.1 (Firmicutes; 33.33%). Additionally, Gemmatimonadota (KCM_1.1; 25.81%), Bacteroidota (KCM_5.2; 23.53%) and Fibrobacterota (KCM_4.2; 22.22%) were defined as sub-dominants in the respective KCM soils. The remaining phyla were distributed unevenly throughout the soils, as Chloroflexota and Planctomycetota were found only in KCM_2.1 (7.14%) and KCM_1.1 (3.23%), respectively.

At the class level, bacterial diversity was presented by 24 (surface soils) and 12 (subsurface soils) classes (Fig. 1B). The dominant phylum Proteobacteria was presented mainly by two classes: Alphaproteobacteria (except KCM_5.2) and Betaproteobacteria (except KCM_4.2). The aforementioned two classes dominated bacterial communities from 15.79% (KCM_4.2) to 69.23% (KCM_2.1) of the total OTUs per given soil. Gammaproteobacteria was distributed only in the soils of KCM_1.1 (3.03%), KCM_5.1 (27.78%) and KCM_5.2 (11.11%), dominating in the structure of KCM_5.1 community together with Betaproteobacteria and Clostridia (phylum Firmicutes) – 77.78% of the total OTUs. Clostridia prevailed also in soils of KCM_4, where it was 52.63% (KCM_4.2) and 34.48% (KCM_4.1). Some classes like Acidimicrobiia (KCM_5.2 and KCM_1.2), Rubrobacteria (KCM_3.1 and KCM_4.1), Gemmatimonadales (KCM_1.1), Chitinophagia (KCM_5.2), Bacilli (KCM_2.2), Chloroflexia (KCM_2.1) and Fibrobacteria (KCM_1.2 and KCM_4.2) manifested preferable local distribution, where they proliferated highly, being from 13.79% to 24.24% of the total community structure. The main classes positively correlated with HMs ($r > 0.70$; $p < 0.03$) were Gemmatimonadales, Micromonosporales, Pseudonocardiales and Planctomycetia, all of them found exclusively in the highly contaminated KCM_1.1.

Identification of 16S rDNA sequences at the species level. A neighbour-joining phylogenetic tree revealed that thirteen bacterial 16S rRNA gene sequences were identified, belonging to Proteobacteria, Actinomycetota and Firmicutes with $\geq 99\%$ homology to their closest relatives, according to BLAST (Fig. 2). The sequence KCM_1_1_49 (OQ422585) had 99.17% similarity to *Massilia neuiana* strain PTW21 (NR_178954.1). This sequence predominated in the surface soils along the gradient of HM contamination, represented by 16 clones. The sequence KCM_1_2_3 (OQ422632) had 99.59% similarity to *Hydrogenophaga palleronii* strain NBRC 102513 (NR_114132.1). It was notably observed in the subsurface soils of KCM_1.2, KCM_2.2 and KCM_5.2 represented by 15 clones. The sequence KCM_4_1_72 (OQ422734) showed 99.52% homology to *Pseudoduganella rivuli* strain FT92W (NR_180844.1). It was represented by 9 clones, distributed in the surface layers of heavily (KCM_4.1) and moderately

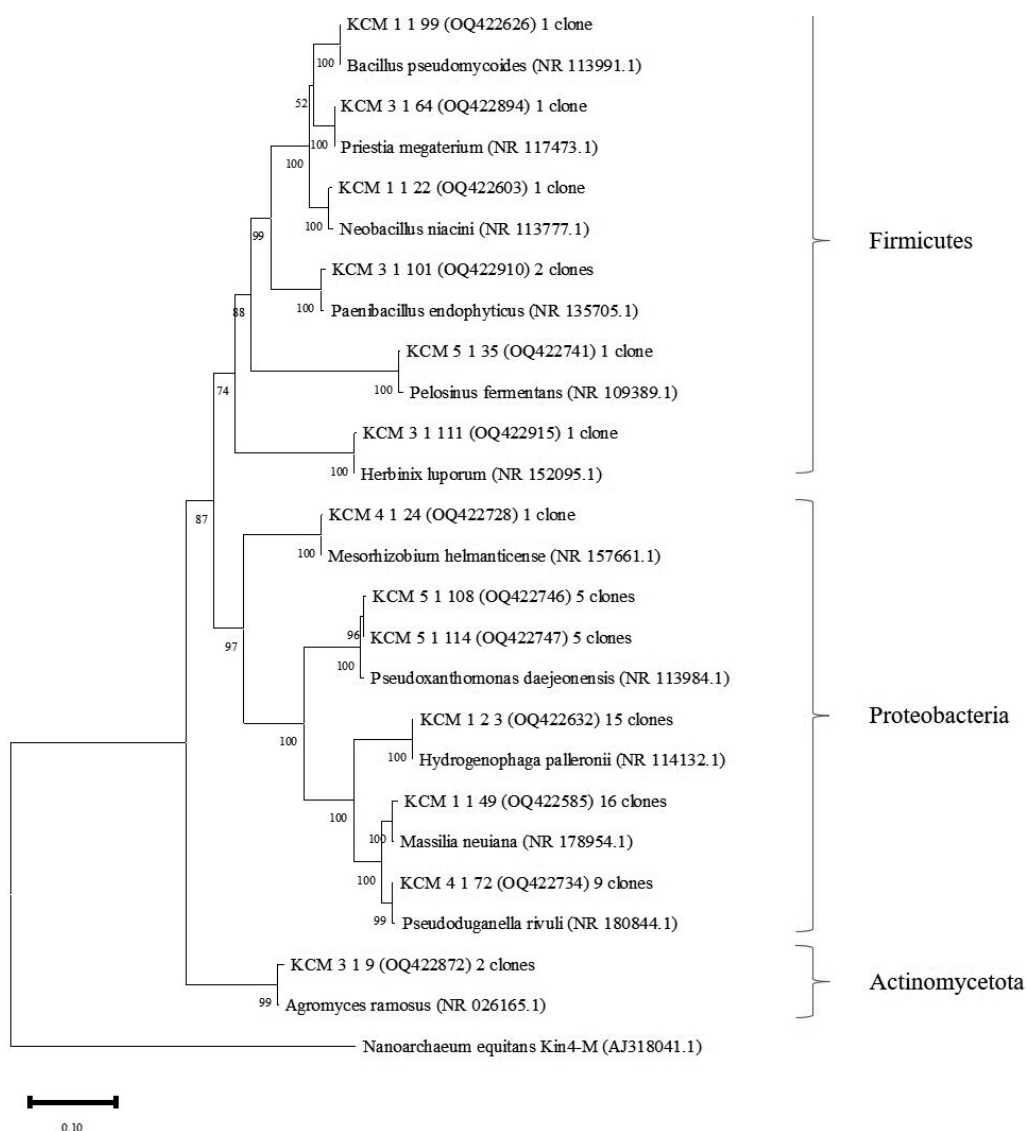


Fig. 2. Neighbour-joining phylogenetic tree based on the 16S rDNA sequences with their reference sequences in GenBank. The tree was rooted with a 16S rDNA sequence of *Nanoarchaeum equitans* strain Kin4-M (AJ318041.1). The scale bar represents ten substitutions per nucleotide position

(KCM_5.1) contaminated soils. The sequences KCM_5_1_108 (OQ422743) and KCM_5_1_114 (OQ422746) showed homology to *Pseudoxanthomonas daejeonensis* strain NBRC 101159 (NR_113984.1) of 99.25% and 99.11%, respectively. They were represented by 5 clones, exclusively in the surface layer of KCM_5. Additionally, KCM_3_1_9 (OQ422872) had a homology of 99.03% to *Agromyces ramosus* strain DMS 43045 (NR_026165.1). It was represented by 2 clones, identified in the slightly contaminated KCM_3.1.

Other identified 16S rDNA sequences, belonging mainly to the Firmicutes species (homology: 99.18–100%), exhibited spot distribution with one to few clones per spot.

Discussion. This study addresses the diversity and structural variations within bacterial communities across the soil HM gradient and soil depth. The used 16S rRNA gene retrieval is generally more robust for analyzing dominant representatives of the bacterial communities and identifying indigenous species.

The relative abundance distribution revealed distinct responses of bacterial phyla to long-term HM contamination. In KCM soils, Proteobacteria, Actinomycetota, and Firmicutes were the most prevalent phyla, identified as dominant or sub-dominant across all bacterial communities. Within Proteobacteria, the classes Alphaproteobacteria and Betaproteobacteria were predominant, exhibiting a wide distribution range (5.56–62.50%) throughout the studied soils. A previous study also highlighted these two classes commonly found in mining soils [14]. Actinomycetota and Firmicutes were represented by six and two classes, respectively. The prevalence of the three phyla was anticipated due to their recognized ecological flexibility and adaptability to thrive in various environments [9,10], including soils contaminated with heavy metals [11]. The representatives of these opportunistic phyla play a crucial role in ecosystems due to their: i) broad metabolic diversity, ii) rapid growth patterns, benefitting from environmental disturbance, and iii) capability to remediate HMs through processes such as bioabsorption, bioleaching, intracellular accumulation, and various enzyme-dependent transformations.

No correlation was found between the HM concentrations and the distribution of taxa, except for the phyla Gemmatimonadota and Planctomycetota, and the classes Micromonosporales, Pseudonocardiales, Gemmatimonadales, and Planctomycetia. The notably high relative abundance of Gemmatimonadota (24.24%; class Gemmatimonadales) in KCM_1.1 highlighted the enrichment of bacteria that are resistant and successfully adapt to HM contamination. The exclusive presence of Gemmatimonadota in heavily contaminated soils was noted also in previous studies [12,13], confirming the particular association of this phylum with HMs. The other classes (Micromonosporales, Pseudonocardiales, and Planctomycetia) were exclusively found in KCM 1.1 soil, comprising between 3.03% and 6.06% of bacterial abundance, and they were defined as tolerant rather than resistant to HMs.

The distribution of taxa by depth was site-specific but statistically insignificant ($p > 0.05$), except for the classes Acidobacteriia and Fibrobacteria, which were exclusively present in surface and subsurface soils, respectively. The prevalence of fibrobacters in deeper soil layer confirmed their preference for anaerobic environments, where they function as cellulose hydrolyzers [15].

The 16S rRNA gene sequences identified at the species level were primarily associated with the phylum Proteobacteria, classes Gammaproteobacteria (*P. dae-*

jeonesis) and Betaproteobacteria (*M. neuiana*, *H. palleronii*, and *P. rivuli*), as well as the phylum Firmicutes, class Bacilli (*P. endophyticus*). These species were distributed across various levels of HM contamination, affirming their ability to thrive in extreme environments, including HM-contaminated ones, owing to the presence of genes encoding resistance to HMs [16,17]. In contrast, 16S rRNA gene sequences affiliated with *A. ramosus* were exclusively found in the soil of slightly polluted KCM 3.1, suggesting its low resistance HMs.

In conclusion, the obtained results emphasize the impact of heavy metal contamination and soil depth on the diversity and structure of bacterial communities, leading to shifts within them. Moreover, they suggest that certain local soil properties may modulate the effects of HMs and influence bacterial responses to the stress factor [6]. To further expand this research, high-throughput sequencing targeting the 16S rRNA gene will be used to explore the complexity of soil microbiome in heavy metal/metalloid contaminated areas.

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