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Structural and functional characteristics of soil microbial communities in response to long-term heavy metal contamination

DISSERTATION SUMMARY

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LIST OF ABBREVIATIONS:

ARDRA – Amplified Ribosomal DNA Restriction Analysis
bp – base pairs
DNA – Deoxyribonucleic acid
rDNA – Ribosomal DNA
AWCD – Average Well Color Development
BLAST – Basic Local Alignment Search Tool
CCA – Canonical Correlation Analysis
CLPP – Community Level Physiological Profile
CFU – Colony-Forming Units
DGGE – Denaturating – gradient gel electrophoresis
dNTP – Deoxyribonucleotide Triphosphates
EDTA - Ethylenediaminetetraacetic Acid
INT - i2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride
IPTG – Isopropyl β-D-1-thiogalactopyranoside
ITS – Internal Transcriber Spacer
KO – KEGG Orthology
KEGG – Kyoto Encyclopedia of Genes and Genomes
LB – Luria- Bertani
NCBI – National Center for Biotechnology and Information
NGS – Next – generation sequencing
NMDS – Non-metric multidimensional scaling
OD – Optical density
OTUs – Operational taxonomic units
PCA – Principal Component Analysis
PCR – Polymerase Chain Reaction
pNP – p-Nitrophenyl
pNPG – p-Nitrophenyl-β-D-glucopyranoside
PGPF – Plant Growth Promoting Fungi
PGPR – Plant Growth Promoting Rhizobacteria
RFLP – Restriction Fragment Length Polymorphism
RISA – Ribosomal Intergenic Spacer Analysis
TBE – Tris–Borate–EDTA Buffer
TE – Tris–EDTA Buffer
TOC – Total organic carbon
HM – Heavy metals/metaloids
UV – Ultraviolet Light
X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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1. Introduction

Soils are a dynamic environment containing a vast diversity of microorganisms that play an essential role in nutrient cycling, the decomposition of organic matter, and the overall soil health.The soil microbiome plays a key role in maintaining ecosystem functions, including metabolic processes and adaptation to various stress factors.

Heavy metals are the main soil pollutants in Bulgaria. This type of contamination causes great concern worldwide, as it represents a serious threat to the environment and human health. Heavy metal pollution alters both the microbial abundance and taxonomic diversity, as well as the function of the soil microbiome. In soils with elevated concentrations of heavy metals, a reduced metabolic activity of sensitive microorganisms is often observed, along with an increase in the relative abundance of resistant and tolerant taxa.

The soil physicochemical properties modify the toxicity of heavy metals and influence the formation and dynamics of microbial communities. Certain physicochemical soil properties – such as pH, organic carbon content, texture, and others – also prove to be significant factors affecting the distribution and activity of specific bacterial and fungal taxa. In this way, the interaction between pollution and local soil characteristics creates selective pressure that determines the dominant microbial taxa in a given soil environment.

The modern approach to studying complex microbial communities is the metagenomic approach, which, in combination with microbiological and molecular-genetic methods, provides a comprehensive understanding of the microbiome structure in soils contaminated with heavy metals. Furthermore, metagenomic analysis allows for an in-depth study of the metabolic capabilities of bacterial communities by predicting their functional potential and specific adaptive mechanisms.

In the scientific literature, numerous studies apply various approaches and methods to investigate the structure and function of microbial communities in soils contaminated with heavy metals. However, the results show that there is no “typical” soil microbiome. For this reason, studying the soil microbiome across different geographical regions and scales – both locally and regionally – is essential for a deeper understanding of microbial diversity and community structure changes.

Up to now, there have been no studies in Bulgaria applying a metagenomic approach to investigate the soil microbiome of soils with long-term heavy metal contamination. The dissertation aims to clarify the influence of heavy metals and specific environmental factors on the structure and function of microbial communities through the application of modern molecular, bioinformatic, and statistical approaches.

2. Aim and objectives

2.1. Aim

The aims of the dissertation are 1) to determine the taxonomic composition and functional activity of the microbiome in soils chronically contaminated with heavy metals, and 2) to identify the key environmental factors influencing the characteristics of microbial communities.

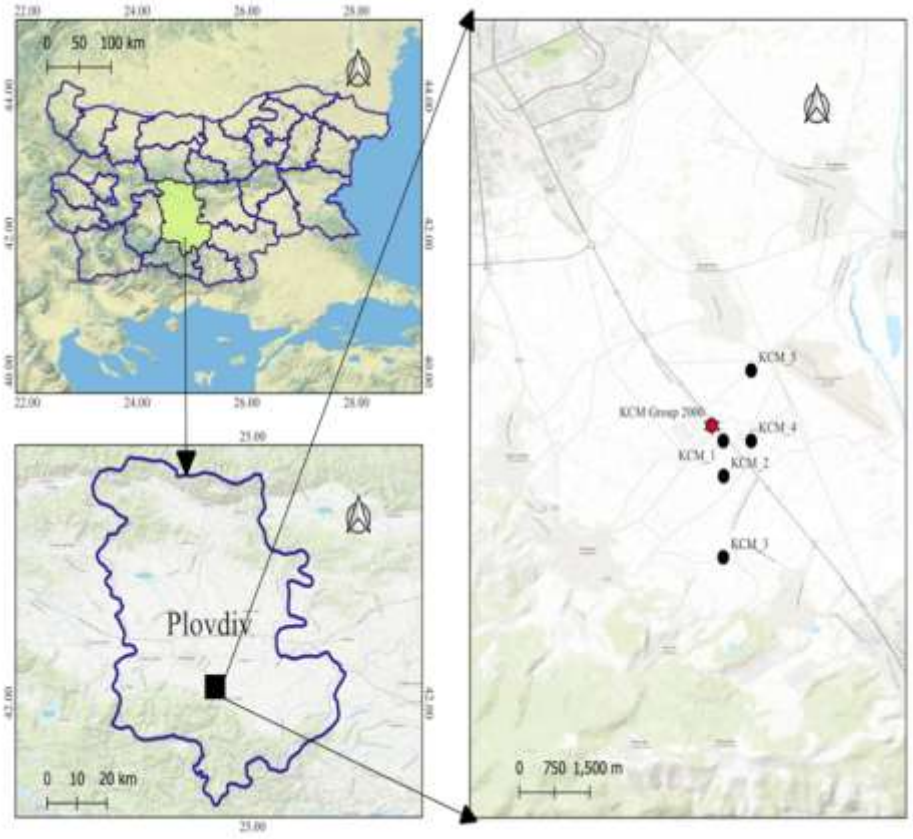
2.2. Objectives

1. To collect soil samples along a gradient of heavy metal contamination (zinc, lead, cadmium, copper) and the metalloid arsenic from the KCM 2000 area, Plovdiv.
2. To determine the soil physicochemical and mechanical properties, as well as the concentrations and bioavailable forms of heavy metals/metalloid.
3. To determine the functional diversity and enzymatic activity of the soil microbial communities.
4. To assess the microbial abundance within the communities.
5. To identify the taxonomic composition of the bacterial and fungal communities.
6. To establish the relationships between the soil physicochemical properties, the concentration of heavy metals/metalloid, and the characteristics of the microbiome.

3. Materials and Methods

3.1. Sampling

Sampling was carried out in June 2020 along a concentration gradient of long-term soil contamination with zinc (Zn), lead (Pb), cadmium (Cd), copper (Cu), and the metalloid arsenic (As) in the area surrounding KCM 2000, Plovdiv. Samples were collected from five geographic locations situated at varying distances from KCM 2000 (Figure 1).



Фигура 1. Geographical location of the sampling points in the area of KCM 2000, Plovdiv.

Soil samples were taken from two depths: 0 – 20 cm (surface soil layer) – KCM 1.1, KCM 2.1, KCM 3.1, KCM 4.1, and KCM 5.1, and 20 – 40 cm (subsurface soil layer) – KCM 1.2, KCM 2.2, KCM 3.2, KCM 4.2, and KCM 5.2. Composite samples were prepared for each point, consisting of five individual cores collected using a soil auger.

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4.1. Soil characteristics

4.1.1. Concentrations of heavy metals/metalloid in soils and level of contamination

In the ten analyzed soils, the presence of Zn, Pb, Cd, Cu, and As was detected, with concentrations exceeding the Maximum allowable concentrations (MAC) according to Regulation No. 3/2008 on the permissible content of harmful substances in soils (eea.government.bg/bg/legislation/soil/normipochvi.doc) (Table 1).

Table 1. Concentrations of heavy metals/metalloid in soils.

Soil	Heavy metal content (mg/kg)				
	Zn	Pb	Cd	Cu	As
KCM_1.1	9452	11569	184.9	891.4	191.0
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	80.0
KCM_5.1	740.0	335.0	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	5.0
KCM_3.2	250.0	130.0	3.6	60.0	9.0
KCM_4.2	4843.1	3424	55.7	341.5	63.5
KCM_5.2	810.0	330.0	9.0	88.2	12.7
***MAC**	320	100	2	150	25

*MAC – Maximum allowable concentrations (MAC) established by Regulation No. 3/2008 on the permissible content of harmful substances in soils. Values exceeding MAC are marked in red, and those below MAC are marked in green.

To determine the level of contamination in the soils, the Nemerow pollution index (NPI) was calculated (Figure 2).

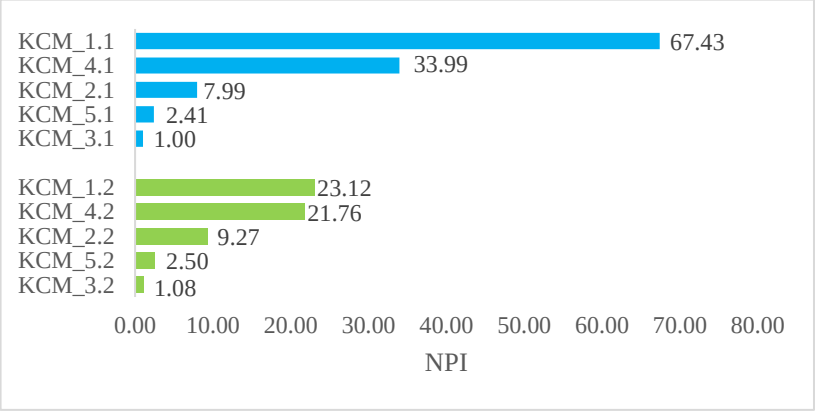


Figure 2. Nemerow pollution index (NPI) of soils

As shown in Figure 2, the NPI values range from 1.00 to 67.43. Accordingly, based on the degree of contamination, the soils can be arranged in ascending order as follows: KCM_3 (slightly contaminated) < KCM_5 (moderately contaminated) < KCM_2 < KCM_4 < KCM_1 (highly contaminated). The surface soil layer (average NPI value: 20.09 ± 25.37) contains higher levels of HMs compared to the subsurface layer (average NPI value: 10.12 ± 8.96). These results are expected, given the well-known low mobility of HMs and their tendency to accumulate predominantly in the surface soil layer.

The bioavailable forms of heavy metals in the soils were determined (Table 2).

Table 2. Values of the bioavailable forms of Pb, Zn, and Cd in soils.

Soil	aPb (mg/kg)	aZn (mg/kg)	aCd (mg/kg)
KCM_1.1	2.6	8.2	9
KCM_2.1	0.2	0.1	0.2
KCM_3.1	0.9	0.1	0.5
KCM_4.1	0.2	1.3	1.1
KCM_5.1	0.4	0.8	0.3
KCM_1.2	0.5	2.4	4.4
KCM_2.2	0.9	0.12	6.4
KCM_3.2	0.8	0.7	0.5
KCM_4.2	0.2	1.7	0.3
KCM_5.2	0.2	0.2	0.2

The KCM_1.1 soil showed the highest concentrations of bioavailable forms of Pb (2.6 mg/kg), Zn (8.2 mg/kg), and Cd (9 mg/kg) compared to the other analyzed soils. The average values of the bioavailable forms across all soils were 0.69 ± 0.73 mg/kg (aPb), 1.56 ± 2.46 mg/kg (aZn), and 2.29 ± 3.18 mg/kg (aCd). These results indicate that the presence of bioavailable forms of Pb, Zn, and Cd varies significantly among the different soils.

4.2. Soil physicochemical properties

4.2.1. Soil mechanical composition

The results indicate that the soils have a sandy-clay texture. In KCM_3 and KCM_5, the soil horizon is continuous, whereas in the other soils there is a distinct difference in percentages between the surface and subsurface soil layers.

4.2.2. Soil pH

Soil pH varies slightly, ranging from slightly acidic: 6.6–6.8 (KCM_4.1, KCM_5.1, and KCM_5.2) to neutral: 7.0–7.2 (the remaining soils) (Table 3). No significant differences in pH with depth were observed, except for KCM_4, where the surface layer is classified as slightly acidic, while the subsurface layer has a neutral pH.

Table 3. Soil physicochemical properties.

Soil	pH	Soil moisture (%)	TOC (g/kg)	NO_3-N (mg/g)	NH_4-N (mg/g)	TOC (mg/g)	P_2O_5 (mg/kg)

KCM_1.1	7.0	16.7	9.65	43.38 ± 0.64	6.62 ± 0.44	50.0	5.69 ± 0.23
KCM_2.1	7.1	12.3	14.07	16.32 ± 0.12	5.13 ± 5.57	21.45	24.02 ± 0.41
KCM_3.1	7.2	9.3	6.45	3.01 ± 0.19	3.26 ± 1.05	6.27	7.42 ± 1.11
KCM_4.1	6.7	14.7	12.33	5.13 ± 0.26	2.25 ± 0.24	7.38	6.89 ± 0.61
KCM_5.1	6.8	22.7	7.035	*ND	2.22 ± 0.32	*ND	*ND
KCM_1.2	7.0	16.7	11.1	19.65 ± 0.21	6.84 ± 0.75	26.49	4.81 ± 0.59
KCM_2.2	7.1	8.7	11.9	5.06 ± 0.51	5.35 ± 0.32	10.41	16.09 ± 0.43
KCM_3.2	7.1	6.7	7.5	4.72 ± 0.29	5.41 ± 0.67	10.13	4.18 ± 0.26
KCM_4.2	7.1	11	16.7	4.42 ± 0.19	3.64 ± 0.4	8.06	10.82 ± 0.27
KCM_5.2	6.6	19.0	7.4	*ND	3.77 ± 0.96	*ND	25.13 ± 1.88

*ND – no data

4.2.3. Soil moisture content

The soil moisture content was determined because it affects the physiological state of soil microbial communities, the solubility of nutrients, and the mobility of heavy metals. Soil moisture varied from 6.7% (KCM_3.2) to 22.7% (KCM_5.1), with slightly higher levels in the surface layer (average 15.14±5.05%) compared to the subsurface layer (average 12.42±5.25%) (table 3).

4.2.4. Total organic carbon content

The lowest total organic carbon content was recorded in KCM_3.1 (6.45%), while the highest content was found in KCM_4.2 (16.7%), as shown in table 4. No significant difference was observed between the mean organic carbon content of the surface layer (9.91±3.29 g/kg) and the subsurface layer (10.92± 3.82g/kg) (F=0.003; p=0.95).

4.2.5. Concentration of inorganic salts

4.2.5.1. Mineral nitrogen

The levels of soluble inorganic nitrogen in the form of nitrate and ammonium ions were analyzed.

The total amount of available inorganic nitrogen was relatively similar among most soils, except for KCM_1, where concentrations were 4-5 times higher than in the other soils (table 4). Elevated levels were also recorded in KCM_2.1. In some soils (KCM_1, KCM_2.1 and KCM_4.1), nitrate nitrogen predominated, representing 69.51% to 86.76% of total inorganic nitrogen, while in the remaining soils the ratio between nitrate and ammonium forms was approximately 50:50.

4.2.5.2. Mineral phosphorus

The highest phosphate contents were observed in soils KCM_2 and KCM_5, while the lowest levels were found in KCM_1 and KCM_3. The surface soil layer contained slightly more available phosphate (14.64±11.08 mg/kg) than the subsurface layer (12.21±8.70 mg/kg), although the difference was not significant (F=0.042; p=0.84).

A principal component analysis (PCA) was performed based on the physicochemical (including heavy metal concentrations) and mechanical soil parameters to determine the similarity of soils in ordination space (figure 3).

PCA indicated that the first two components explained 82.14% of the total variation, with PC 1 accounting for 67.16% and PC 2 for 14.98%. The HM content (total and bioavailable concentrations) contributed most strongly to the variability described by PC1.

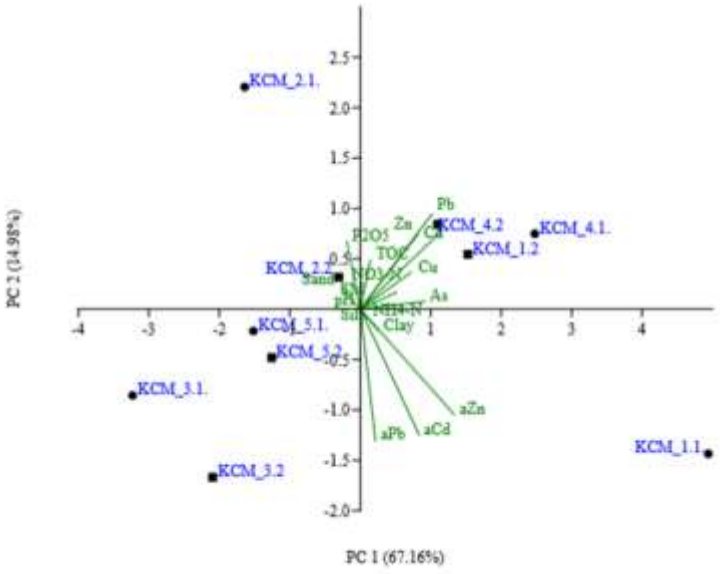


Figure 3. Principal component analysis (PCA) based on the physicochemical and mechanical properties of the studied soils.

The main sources of variation along PC2 are the total organic carbon (TOC) content and the concentrations of the bioavailable forms of Pb (aPb) and Cd (aCd).

The two principal components separate the soils into distinct groups as follows: lower-left quadrant: KCM_3 and KCM_5 (1<NPI<2.5), upper-left quadrant: KCM_2 (7.99<NPI<9.27). upper-right quadrant: KCM_4 and KCM_1.2 (21.76<NPI<33.99), lower-right quadrant: KCM_1.1 (NPI=67.43) The distinct separation of KCM_1.1 from the other highly contaminated soils is associated with its extremely high NPI values, as well as the elevated concentrations of bioavailable Pb, Zn, and Cd.

4.3. Determination of bacterial abundance

To assess bacterial abundance in soils along the heavy metal (HM) contamination gradient, two approaches were applied: 1) culturable method, based on determining colony-forming units (CFU), which reflects the number of viable bacteria present in the soils, and 2) molecular-genetic method, quantifying the number of 16S rRNA gene copies via quantitative PCR (qPCR), which provides an estimate of the total bacterial abundance. Both methods are important for evaluating soil health and productivity, as well as for determining the influence of environmental factors on bacterial abundance along the contamination gradient.

4.3.1. Bacterial abundance expressed as colony-forming units (CFU)

Figure 4 presents the results of bacterial abundance determined via CFU counts.The abundance of culturable heterotrophic bacteria in the surface soil layer is distributed as follows: KCM_2.1 ($3.7\pm0.19 \times 10^6$

CFU/g soil), KCM_3.1 (2.91±0.25 x10^6 CFU/g soil), KCM_5.1 (1.6±0.51 x10^6 CFU/g soil), KCM_4.1 (1.55±0.33 x10^6 CFU/g soil) и KCM_1.1 (1.3±0.34 x10^6 CFU/g soil). In the subsurface soil layer, the highest abundance of culturable heterotrophic bacteria is observed in the slightly contaminated soil KCM_3.2 (4.5±0.79 x10^6 CFU/g soil), followed by KCM_4.2 (3.28±1.33 x10^6 CFU/g soil), KCM_5.2 (3.11±0.21 x10^6 CFU/g soil), KCM_2.2 (2.1±0.00 x10^6 CFU/g soil) and KCM_1.2 (1.44±0.14 x10^6 CFU/g soil).

When comparing the two sampling depths, the subsurface layer (2.89±0.45 x10^6 CFU/g soil) contains a higher abundance of culturable heterotrophic bacteria than the surface layer (2.21±0.32 x10^6 CFU/g soil). A pronounced decrease in bacterial abundance is observed in the highly contaminated soils: KCM_1.1 shows a 41.4% reduction relative to the mean CFU value for the surface layer, while KCM_1.2 shows a 50.2% reduction relative to the mean for the subsurface layer.

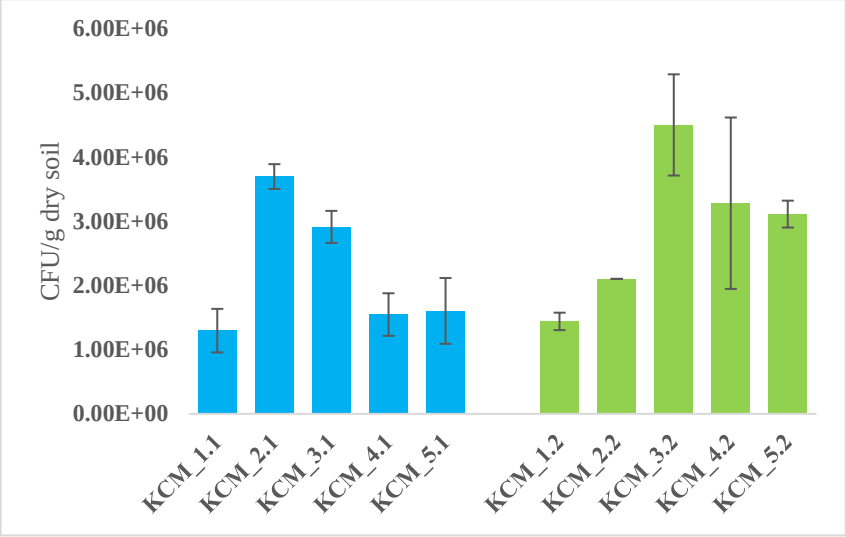


Figure 4. Bacterial abundance of culturable heterotrophic bacteria in the surface (blue) and subsurface (green) soil layers.

4.3.2. Bacterial abundance determined by quantitative PCR (qPCR)

Figure 5 presents the results of bacterial abundance determined by quantitative PCR (qPCR). In the surface soil layer, the highest abundance of 16S rRNA gene copies was detected in the highly contaminated soil KCM_2.1 (1.78±0.18 x10^{11}), followed by KCM_3.1 (1.15±0.10 x10^{11}) and KCM_5.1 (0.95±0.12 x10^{11}). Lower bacterial abundance was observed in the highly contaminated soils KCM_4.1 (0.41±0.025 x10^{11}) and KCM_1.1 (0.14±0.11 x10^{11}). In the subsurface soil layer, the highest 16S rRNA gene copy numbers were also found in the highly contaminated soil KCM_2.2 (1.95±0.44 x10^{11}). The remaining soils in this layer exhibited lower abundance, as follows: KCM_4.2 (0.41±0.04 x10^{11}), KCM_5.2 (0.28±0.2 x10^{11}), KCM_3.2 (0.27±0.05 x10^{11}), KCM_1.2 (0.26±0.09 x10^{11}).

When comparing the two soil layers, higher bacterial abundance was observed in the surface layer (average 8.91±0.10x10^{10} copies/g soil), than in the subsurface layer (average 6.38±0.14x10^{10} copies/g soil), which is opposite to the trend observed using the culture-based method. The bacterial abundance decreased in the highly contaminated soils KCM_1.1 (83.34% reduction relative to the other surface soils) and KCM_1.2 (58.92% reduction relative to the other subsurface soils).

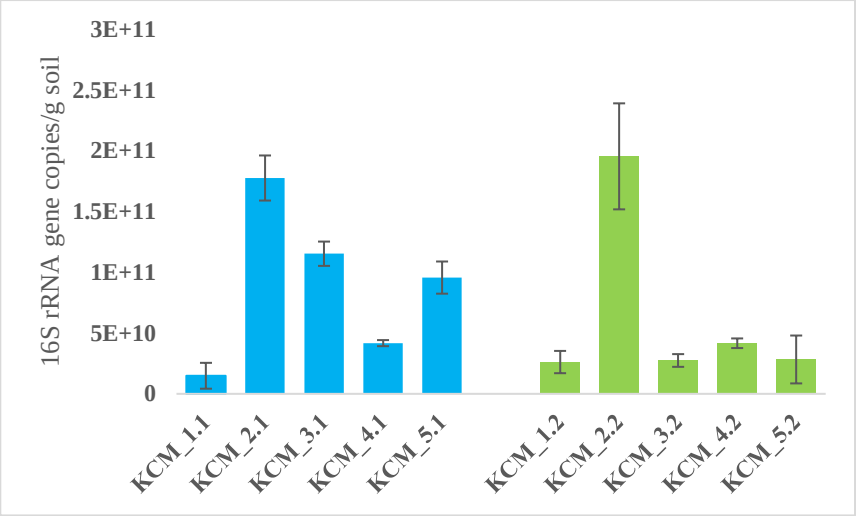


Figure 5. Bacterial abundance expressed as 16S rRNA gene copy numbers in the surface (blue) and subsurface (green) soil layers.

4.3.3. Correlation relationships between bacterial abundance and soil abiotic characteristics

To determine which abiotic soil properties influence the distribution of bacterial abundance (culturable heterotrophic bacteria and 16S rRNA gene copy numbers), a Pearson linear correlation analysis was performed. The results are presented in figure 6.

The calculated correlation relationships were not statistically significant ($p > 0.05$). Nevertheless, for both surface and subsurface soil layers, the correlation analysis showed a moderate positive correlation between bacterial abundance (CFU and 16S rRNA gene copies) and soil pH ($r=0.39$ and $r=0.33$, respectively), as well as with phosphate (P_2O_5) concentration ($r=0.39$ and $r=0.39$, respectively).

A relatively strong negative correlation was found between bacterial abundance (CFU and 16S rRNA gene copies) and HM concentrations in the soils (r ranging from -0.40 to -0.60). A negative correlation was also observed between bacterial abundance (CFU) and soil moisture (SM; $r = -0.60$) and nitrate nitrogen (NO_3–N; $r = -0.39$). The 16S rRNA gene copy numbers showed a moderate negative correlation with the content of silt particles in the soil ($r = -0.36$).

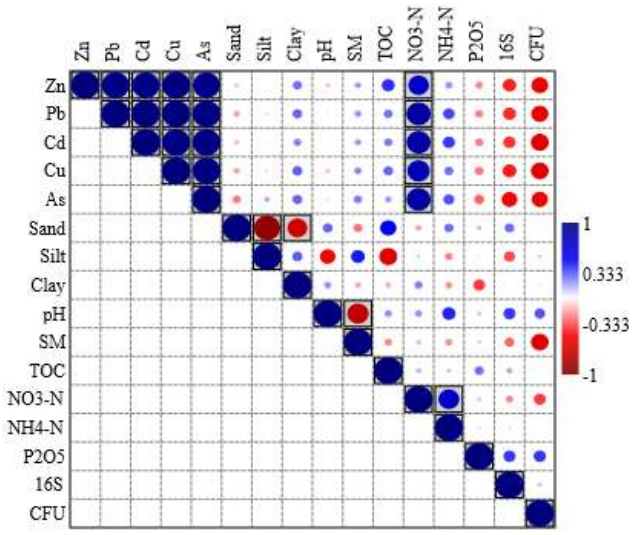


Figure 6. Pearson linear correlation relationships between bacterial abundance (CFU and 16S rRNA gene copies) and soil abiotic characteristics. The size of the circles reflects the strength of the correlation, while those enclosed in squares represent significant correlations.

The correlation coefficients tend to be higher (although statistically insignificant) between CFU values and HM concentrations compared to those between 16S rRNA gene copies and HMs. This likely indicates a stronger effect of contamination on the culturable (actively metabolizing) bacteria at the time of sampling, compared to the total bacterial community, which includes both culturable and non-culturable species.

4.4. Taxonomic composition and structure of bacterial communities

The taxonomic composition and structure of the bacterial communities were determined using two molecular approaches: 1) construction of 16S rRNA gene clone libraries, and 2) targeted amplicon sequencing of the 16S rRNA gene

4.4.1. Diversity and ctructure of bacterial communities determined by 16S rRNA gene clone library construction

The construction of 16S rRNA gene clone libraries is a molecular technique used to identify the dominant representatives of bacterial communities, allowing their identification at the species level. This is achieved through cloning the full-length 16S ribosomal RNA gene (~1500 bp) into an *E. coli* vector. Subsequent sequencing of the cloned 16S rRNA genes provides data for the phylogenetic affiliation of the identified bacterial species by comparison with reference sequences from public databases (NCBI GenBank).

4.4.2. Characteristics of the 16S rRNA gene clone libraries

Nine 16S rRNA gene clone libraries were constructed from the soil samples. The library for KCM_3.2 could not be obtained due to unsuccessful attempts to clone the PCR product into the vector. Statistical information for all available clone libraries is summarized in table 4.

Table 4. Statistical summary of the 16S rRNA gene clone libraries

Soil	Total clones	No. of OTUs	RFLP/*Msp*I	Sequenced clones
KCM_1.1	109	42	81	57
KCM_2.1	109	54	71	13
KCM_3.1	104	60	83	56
KCM_4.1	105	46	71	10
KCM_5.1	112	68	82	9
KCM_1.2	104	60	84	8
KCM_2.2	100	63	74	7
KCM_4.2	108	56	68	2
KCM_5.2	101	56	72	4
Total	**952**	**505**	**686**	**166**

The constructed clone libraries contained a total of 952 clones, of which 686 were visually grouped based on their RFLP profiles into 505 OTUs.The number of OTUs per library ranged from 42 (KCM_1.1) to 68 (KCM_5.1). A total of 166 clones were selected for sequencing based on their RFLP profiles. After Sanger sequencing and sequence processing, 166 reliable partial 16S rDNA sequences were annotated in the GenBank database under accession numbers OQ422575–OQ422639; OQ422707–OQ422751; OQ422861–OQ422916.

The taxonomic resolution level of the sequences was determined as follows:

- **Species level:** 9.04% of all sequences showed 99-100% homology with their closest relative and were identified to species level (the highest number was found in soils KCM_3.1 and KCM_5.1).
- **Genus level:** 19.28% of sequences showed 97-98% homology and were identified to genus level.
- **Class level:** 47.59% of sequences showed 90-96% homology and were classified to class level.
- **Phylum level:** 24.10% of sequences showed <90% homology and were classified only to phylum level.

The soil bacterial diversity indicates the presence of previously uncultured bacterial taxa, as 24.10% of all sequences exhibited less than 90% similarity to known reference 16S rRNA gene sequences in the NCBI database.

4.4.3. Alpha diversity of bacterial communities in clone libraries

Data from the clone libraries were used to calculate the richness indices (Chao1 and ACE) and the alpha diversity index (Shannon) of the soil bacterial communities, and the results are presented in table 5.

According to the values of the richness indices Chao1 and ACE, soils KCM_4.2 (Chao1: 1414; ACE: 1138) and KCM_5.1 (Chao1: 1033; ACE: 742.4) exhibit a greater bacterial community richness. The Shannon diversity index values vary only slightly among the soils, ranging from 3.708 (KCM_1.1) to 4.494 (KCM_5.1). The highly contaminated soil KCM_1.1 is characterized by the lowest values of all three indices (Chao1: 77.67; ACE: 93.93; Shannon: 3.708), indicating a more limited and uniform bacterial community.

Table 5 Values of richness (Chao1, ACE) and alpha diversity (Shannon) indices in the studied soils.

Soil	Chao1	ACE	Shannon
KCM_1.1	78	94	3.708
KCM_2.1	258	288	4.207
KCM_3.1	162	203	4.301
KCM_4.1	119	201	3.897
KCM_5.1	1033	742	4.494
KCM_1.2	766	504	4.237
KCM_2.2	470	715	4.466
KCM_4.2	1414	1138	4.186
KCM_5.2	334	262	4.314

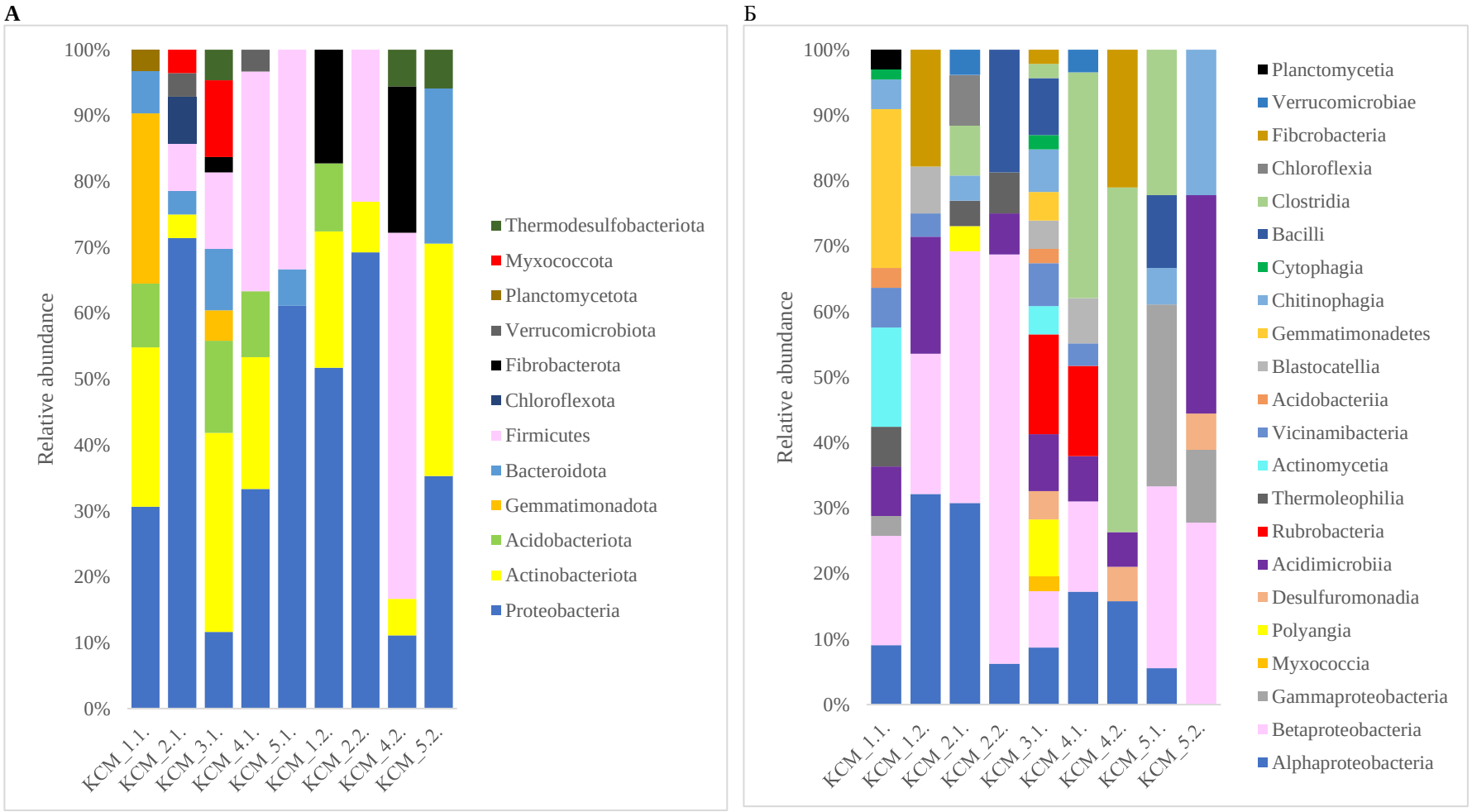
4.4.4. Taxonomic diversity of bacterial communities at the phylum and class levels

The bacterial community diversity was represented by 12 phyla in the surface and 7 phyla in the subsurface soil layers (figure 7A). In all studied soils, the dominant phylum was Proteobacteria (30.65%–71.43% of total abundance), except in KCM_3.1 (11.63%) and KCM_4.2 (11.11%), where Actinobacteriota (KCM_3.1: 30.23%) and Firmicutes (KCM_4.2: 55.56%) were dominant, respectively. Gemmatimonadota (KCM_1.1: 25.81%), Bacteroidota (KCM_5.2: 23.53%), and Fibrobacterota (KCM_4.2: 22.22%) were subdominant in the corresponding soils.

Proteobacteria (11.11-71.43%), Actinobacteriota (up to 35.29%), Acidobacteriota (up to 13.95%), Bacteroidota (up to 23.53%), Firmicutes (up to 55.56%), Fibrobacterota (up to 22.22%), and Thermodesulfobacteriota (up to 5.88%) were distributed across both soil layers. The phyla Gemmatimonadota, Chloroflexota, Verrucomicrobiota, Planctomycetota, and Myxococcota were present only in the surface soil layer of KCM_2.1, KCM_3.1, and KCM_4.1. The remaining phyla were unevenly distributed among all soils, with Chloroflexota and Planctomycetota detected only in KCM_2.1 (7.14%) and KCM_1.1 (3.23%), respectively.

At the class level, bacterial diversity was represented by 24 classes in the surface and 12 classes in the subsurface soil layer (figure 7B). The dominant class across all soils was Alphaproteobacteria (except in KCM_5.2), followed by Betaproteobacteria (except in KCM_4.2). Alphaproteobacteria dominated the bacterial communities, ranging from 5.56% (KCM_5.1) to 32.14% (KCM_1.2). Betaproteobacteria ranged from 8.70% (KCM_3.1) to 62.50% (KCM_2.2). In KCM_5.1, the dominant classes were Gammaproteobacteria (27.78%), Betaproteobacteria (27.78%), and Clostridia (34.48%). Clostridia also prevailed in the highly contaminated soils KCM_4.1 (34.48%) and KCM_4.2 (52.63%).

The classes Acidimicrobiia (KCM_5.2 and KCM_1.2), Rubrobacteria (KCM_3.1 and KCM_4.1), Gemmatimonadetes (KCM_1.1), Chitinophagia (KCM_5.2), Bacilli (KCM_2.2), Chloroflexia (KCM_2.1), and Fibrobacteria (KCM_1.2 and KCM_4.2) showed preference for specific soils, comprising 13.79%-24.24% of the relative bacterial abundance. The main classes found exclusively in the highly contaminated soil KCM_1.1 were Gemmatimonadetes and Planctomycetia.



Фигура 7. Relative abundance of bacterial phyla (A) and classes (B) in the studied soils based on the sequenced 16S rDNA fragments.

4.4.5. Phylogenetic diversity of bacterial communities at the species level

The phyronic diversity of the soils was assessed based on 16S rDNA sequences showing ≥99% homology with their closest relatives according to the NCBI database, and were thus identified to the species level (figure 8).

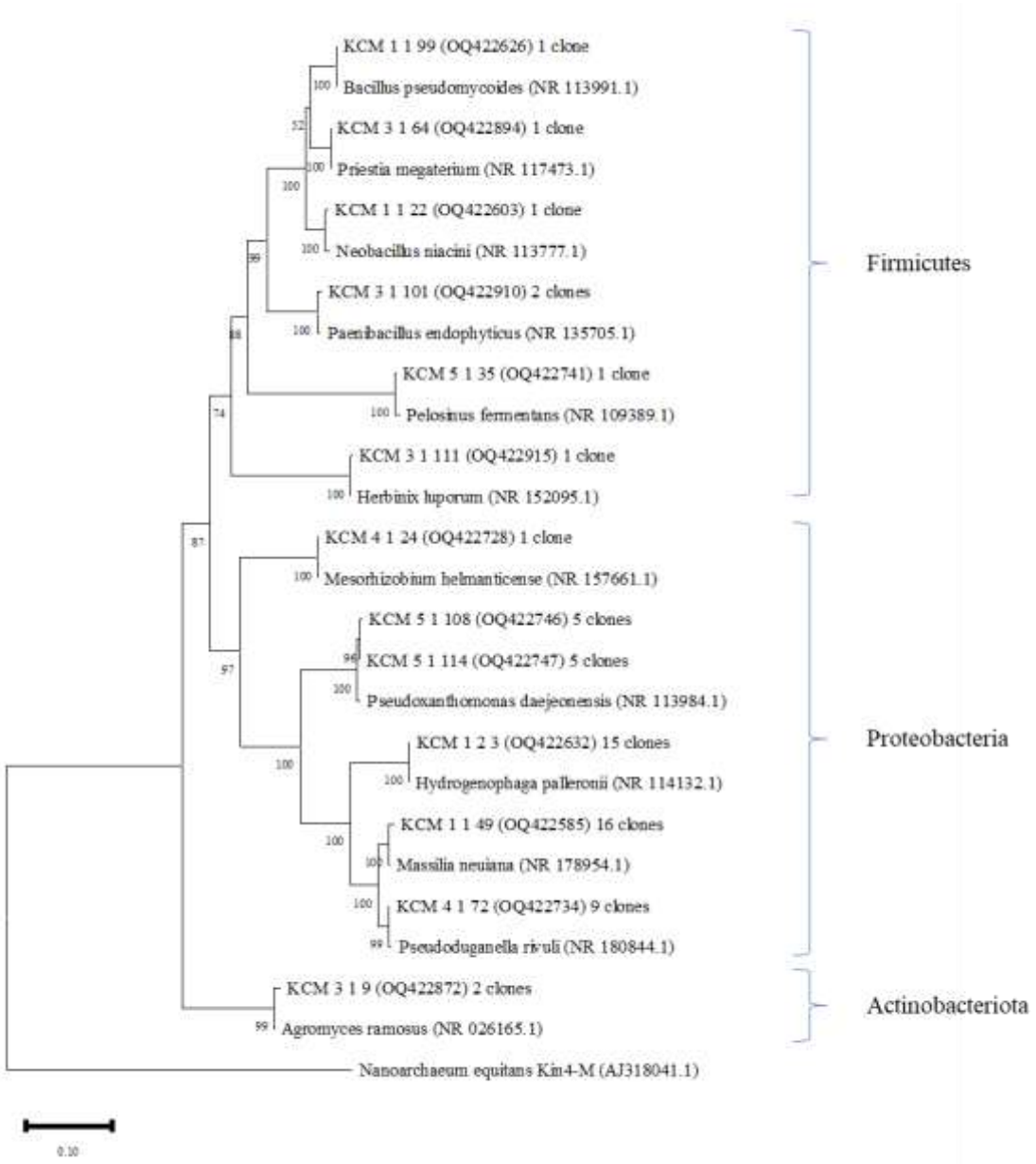


Figure 8. Neighbor-joining phylogenetic tree based on 16S rDNA sequences with ≥99% similarity, showing evolutionary distances between species of 0.10. The tree is rooted, with the 16S rDNA sequence of *Nanoarchaeum equitans* strain Kin4-M (AJ318041.1) used as an outgroup.

A total of 13 bacterial species were identified, belonging to three main phyla: Proteobacteria (6 sequences), Actinobacteriota (6 sequences), and Firmicutes (1 sequence).

The sequence KCM_1_1_49 (OQ422585) was the most abundant, represented by 16 clones in surface soils. It showed 99.17% similarity with *Massilia neuiana*, strain PTW21 (NR_178954.1).

The sequence KCM_1_2_3 (OQ422632) was represented by 15 clones identified in the subsurface soils of KCM_1.2, KCM_2.2, and KCM_5.2, showing 99.59% similarity with *Hydrogenophaga palleronii*, strain NBRC 102513 (NR_114132.1).

The sequence KCM_4_1_72 (OQ422734) included 9 clones found in the surface layers of both highly contaminated (KCM_4.1) and moderately contaminated (KCM_5.1) soils. It displayed 99.52% homology with *Pseudoduganella rivuli*, strain FT92W (NR_180844.1).

Sequences KCM_5_1_108 (OQ422743) and KCM_5_1_114 (OQ422746) each contained 5 clones, identified in the surface layer of KCM_5. They showed 99.25% and 99.11% similarity, respectively, with *Pseudoxanthomonas daejeonesis*, strain NBRC 101159 (NR_113984.1).

The sequence KCM_3_1_9 (OQ422872) was represented by 2 clones identified in the slightly contaminated soil KCM_3.1, with 99.03% similarity to *Agromyces ramosus*, strain DSM 43045 (NR_026165.1).

The remaining identified 16S rDNA sequences were represented by 1 to 2 clones each and mainly belonged to species from the phylum Firmicutes, showing 99.18%-100% homology.

4.5. Diversity and structure of bacterial communities based on targeted amplicon sequencing of the 16S rRNA gene

Targeted amplicon sequencing of the 16S rRNA gene is based on the amplification of a small fragment from the hypervariable regions of the gene - in this study, the V3-V4 region (~385 bp) - followed by sequencing on an Illumina MiSeq platform.

4.5.1. Alpha diversity of bacterial communities based on targeted amplicon sequencing of the 16S rRNA gene

A total of 181136-196090 paired-end reads of the hypervariable V3-V4 region of the 16S rRNA gene were obtained for the ten soil samples. These sequences were clustered into OTUs, ranging from 324 (KCM_3.2) to 557 (KCM_1.2). Based on these data, the alpha diversity indices (Shannon and Simpson) were calculated in table 6.

The highest number of OTUs and bacterial community diversity were observed in the most contaminated soils, KCM_1.1 and KCM_1.2. In contrast, the slightly contaminated soil KCM_3.2 showed the lowest OTU count (324) and lowest Shannon index (7.916).

Table 6. Characteristics and alpha diversity indices of surface and subsurface soils based on targeted amplicon sequencing of the 16S rRNA gene.

Soil	Sequences		Diversity indices	
	Reads	Total OTUs	Shannon	Simpson
KCM_1.1	165732	520	8.591	0.996
KCM_2.1	168680	466	8.417	0.996
KCM_3.1	180510	507	8.518	0.996

KCM_4.1	174644	531	8.468	0.996
KCM_5.1	165606	436	8.203	0.995
KCM_1.2	181136	557	8.625	0.996
KCM_2.2	151890	376	7.958	0.994
KCM_3.2	175612	324	7.916	0.998
KCM_4.2	196090	471	8.333	0.996

4.5.2. Taxonomic composition of bacterial communities at the phylum and class levels based on targeted amplicon sequencing of the 16S rRNA gene

The relative taxonomic abundance of the bacterial communities was represented by a total of 15 phyla (figure 9A). The dominant phylum in all soils was Proteobacteria (19.13-39.13%), followed by Actinobacteriota (10.41-31.15%) and Acidobacteriota (5.84-19.23%). The subdominant phyla across the soils included Bacteroidota (2.29-13.14%), Chloroflexota (2.78-11.2%), and Gemmatimonadota (1.91-9.71%). The phyla Gemmatimonadota and Planctomycetota were particularly abundant in the highly contaminated soils KCM_1.1 (9.71% and 9.84%) and KCM_1.2 (8.47% and 8.36%). In moderately contaminated soils (KCM_5.1 and KCM_5.2), the relative abundance of Proteobacteria (37.07-39.13%) and Bacteroidota (10.51-13.14%) increased, whereas Acidobacteriota decreased with depth (5.84–7.63%). Chloroflexota was most prevalent in KCM_4.1 (11.2%) and KCM_1.2 (8.42%), while Verrucomicrobiota showed the highest abundance in KCM_2.1 (5.16%) and KCM_4.2 (4.83%).

The taxonomic diversity at the class level included a total of 27 classes (figure 9B). The most dominant class across all soils was Alphaproteobacteria (8.62-21.23%), followed by Gammaproteobacteria (8.01-18.37%) and Actinobacteria (3.68-11.53%). The subdominant classes in the community structure were Thermoleophilia (1.63-14.48%), Vicinamibacteria (3.06-12.64%) and Bacteroidia (2.29-13.11%). The classes Gemmatimonadetes (8.47-9.71%) and Planctomycetia (3.19-3.71%) were identified mainly in the highly contaminated soils (KCM_1.1 and KCM_2.1). In moderately contaminated soils (KCM_5.1 and KCM_5.2), a higher abundance of Alphaproteobacteria (18.7-21.23%), Gammaproteobacteria (17.89-18.37%), Bacilli (5.94-8.03%), and Bacteroidia (10.42-13.11%) was observed. The classes Vicinamibacteria (10.6-11.1%) and Thermoleophilia (8.62-14.48%) were more prominent in the slightly contaminated soils.

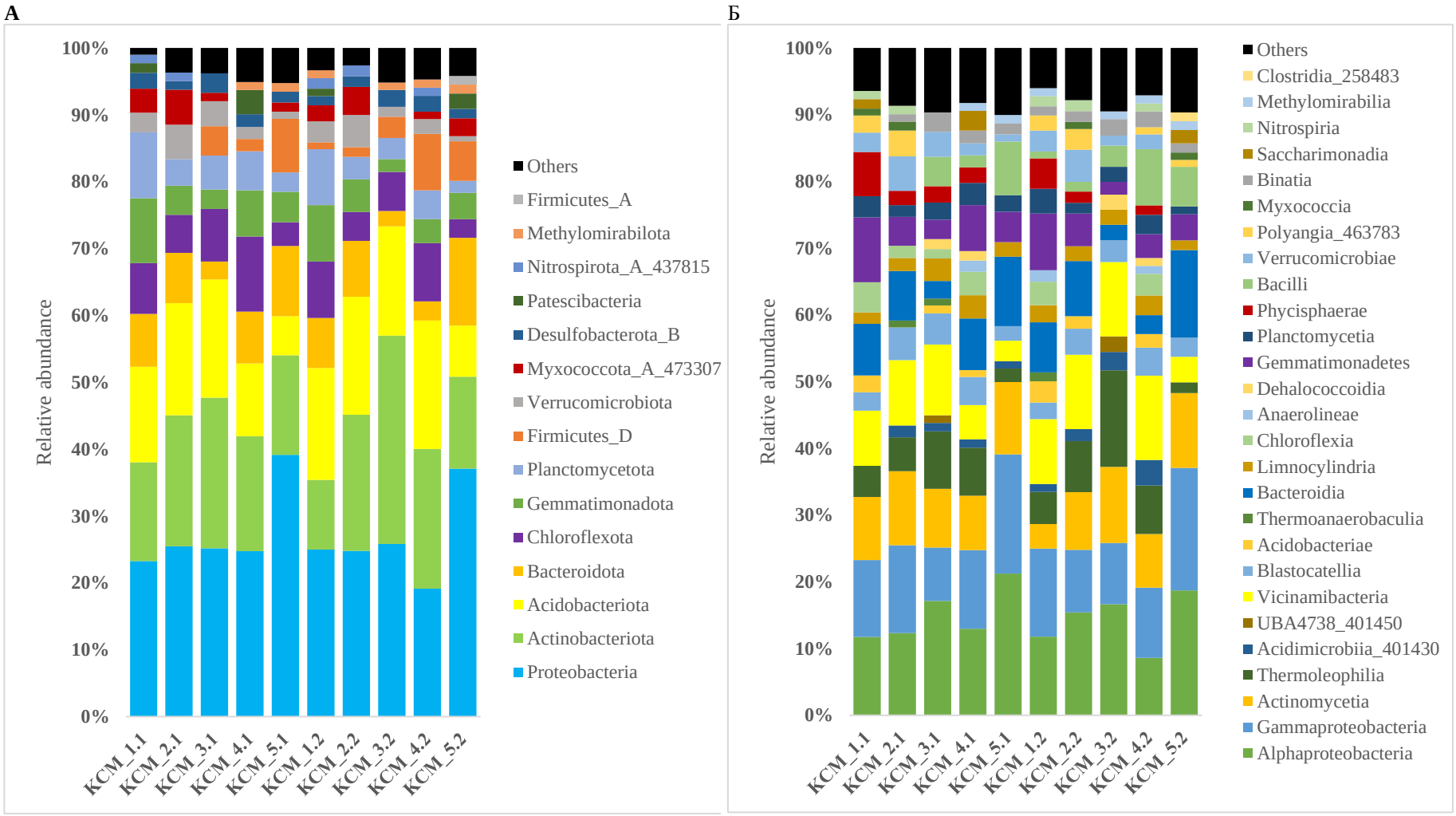


Figure 9. Relative abundance of bacterial community phyla (A) and classes (B) in the studied soils based on 16S rRNA gene amplicon sequencing. "Others" represent unclassified taxa and taxa with relative abundance <1%.

4.5.3. Taxonomic composition of bacterial communities at the genus level based on targeted amplicon sequencing of the 16S rRNA gene

Bacterial abundance at the genus level was represented by 43 genera belonging to different classes and phyla. Due to the high proportion of unclassified genera (61.44-74.32%), the analysis focused on the 20 most abundant bacterial genera, presented in figure 10.

The genera *Gp6-AA45* and *PSRF01* (>1%) were detected in all soils along the contamination gradient. In the most highly contaminated soil (KCM_1), the genera UBA2421 (Phycisphaerae, Planctomycetota), *AG11* (Gemmatimonadetes, Gemmatimonadota) and *Sulfotelmatobacter* (Acidobacteriae, Acidobacteriota) were identified. The genus *Aquicella_A* (Gammaproteobacteria, Proteobacteria) was found exclusively in the highly contaminated soil KCM_4 (NPI=27.88). In the moderately contaminated soil (KCM_5), the genera *JAABRT01* (Gemmatimonadetes, Gemmatimonadota), *Mycobacterium* (Actinomycetia, Actinobacteriota), *Devosia_A_501803*, *Devosia_A_502124* (Alphaproteobacteria, Proteobacteria), and *Nitrosospira* (Gammaproteobacteria, Proteobacteria) were detected. The slightly contaminated soil KCM_3 was characterized by the presence of the genera *AC-16* (Thermoleophilia, Actinobacteriota), *Solirubrobacter* (Gemmatimonadetes, Gemmatimonadota), *Sphingomicrobium_483265*, *HRBIN40* (Alphaproteobacteria, Proteobacteria), and *AC-51* (UBA4738_401450, Actinobacteriota).

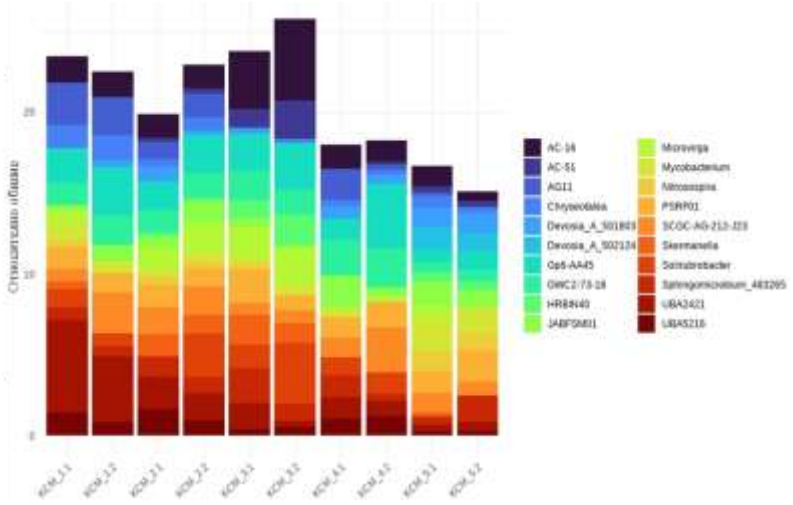


Figure 10. Taxonomic composition of bacterial communities at the genus level. The 20 most abundant bacterial genera are presented.

4.5.4. Correlations between taxonomic diversity at the class level and heavy metals

To visualize the similarity of bacterial communities based on the relative abundance of bacterial classes, a Multidimensional Scaling (MDS) analysis was performed (figure 11).

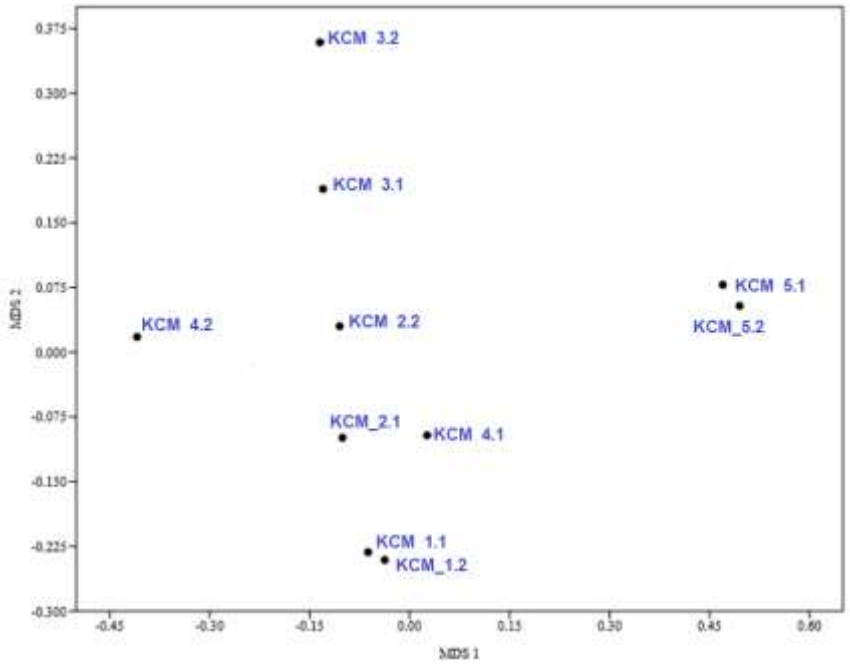


Figure 11. MDS plot showing the taxonomic similarity of soil bacterial communities at the class level using the Euclidean similarity index. Stress = 0.079.

High taxonomic similarity was observed between soils KCM_2 and KCM_4, characterized by contamination levels ranging from NPI=7.99 (KCM_2.1) to NPI=33.99 (KCM_4.1). The remaining soils formed distinct groups, which were dissimilar both from each other and from the aforementioned pair.

To assess which of the two main factors (contamination level and soil depth) influenced the distribution of bacterial classes, a two-way Permutational multivariate analysis of variance (PERMANOVA) was conducted (table 7).

Table 7. Results of two-way PERMANOVA for the effect of heavy metal contamination level (NPI) and soil depth on the distribution of bacterial classes.

Source of variation	Sum of squares	Degree of freedom	Mean square	F	p
NPI	2269.87	4	567.47	1796.1	0.0001
Depth	121.91	1	121.91	385.87	0.0001
NPIxDepth interaction	426.033	4	106.51	337.12	0.0001
Residuals	6.31876	20	0.31594		
Total	2824.1	29			

PERMANOVA confirmed the significant effect of contamination (F=1796; p=0.0001) and soil depth (F=356; p=0.0001) on the distribution of bacterial classes. The interaction between these two factors was also statistically significant (F=337; p=0.0001). Together, the two factors explained more than 80% of the total variation in bacterial community composition.

To identify the bacterial classes contributing most to dissimilarity, a SIMPER analysis was performed (table 8).

Таблица 8. Results of SIMPER analysis showing the contribution of bacterial classes to the overall dissimilarity (29.05%) of soil bacterial communities.

Class	Average dissimilarity	Individual contribution (%)	Cumulative Contribution (%)
Alphaproteobacteria	2.64	9.09	9.09
Thermoleophilia	2.42	8.32	17.41
Gammaproteobacteria	2.37	8.15	25.56
Bacteroidia	2.34	8.05	33.61
Vicinamibacteria	2.23	7.67	41.28
Bacilli	2.02	6.97	48.24
Gemmatimonadetes	1.66	5.71	53.95
Actinomycetia	1.41	4.83	58.79
Phycisphaerae	1.37	4.72	63.50
Chloroflexia	1.21	4.15	67.66
Verrucomicrobiae	1.15	3.97	71.62
Polyangia_463783	0.99	3.42	75.04
Acidobacteriae	0.78	2.68	77.73
Acidimicrobiia_401430	0.72	2.46	80.19
Blastocatellia	0.65	2.25	82.44
Planctomycetia	0.56	1.94	84.38
Saccharimonadia	0.56	1.92	86.30
Dehalococcoidia	0.55	1.89	88.19
Binatia	0.54	1.85	90.04
Nitrospiria	0.47	1.63	91.66
Limnocylindria	0.45	1.54	93.21
Anaerolineae	0.42	1.46	94.66
Methylomirabilia	0.39	1.34	96.01
UBA4738_401450	0.39	1.33	97.33
Myxococcia	0.35	1.20	98.53
Thermoanaerobaculia	0.29	0.98	99.51
Clostridia_258483	0.14	0.49	100.00

The classes Alphaproteobacteria, Thermoleophilia, Gammaproteobacteria, Bacteroidia, Vicinamibacteria and Bacilli were identified as having a significant role in explaining community dissimilarities, together accounting for approximately 53% of the total variation.

The variability in the taxonomic composition of the bacterial communities is influenced not only by contamination level and soil depth but also likely by other environmental factors. Pearson correlation analysis was conducted to determine which bacterial classes were sensitive to HM contamination and soil depth.

Significant correlations were observed between NPI and several bacterial classes, including Alphaproteobacteria ($r=-0.56$; $p=0.0005$), UBA4738_401450 ($r=-0.34$; $p=0.041$), Acidobacteriae ($r=0.63$; $p=0.0002$), Chloroflexia ($r=0.85$; $p<0.001$), Gemmatimonadetes ($r=0.84$; $p<0.0001$), Planctomycetia ($r=0.61$; $p=0.0004$), Phycisphaerae ($r=0.83$; $p<0.0001$), Binatia ($r=-0.58$; $p=0.022$), Bacilli ($r=-0.36$; $p=0.048$), Saccharimonadia ($r=0.45$; $p=0.013$) and Nitrospiria ($r=0.39$; $p=0.033$). Most of these correlations were positive, indicating a degree of tolerance of these bacterial classes to long-term HM contamination. Negative correlations, on the other hand, suggest a suppressive effect of contamination on the corresponding taxa. According to Pearson correlation results, soil depth had a lesser influence on class-level distribution. Significant positive correlations were found between soil depth and Acidimicrobiia_401430 ($r=0.38$; $p=0.04$), Vicinamibacteria ($r=0.36$; $p=0.047$) and Methylomirabilia ($r=0.40$; $p=0.03$). All correlation coefficients are positive, suggesting that representatives of these classes prefer habitats with lower oxygen content.

4.6. Taxonomic composition and structure of the fungal communities

4.6.1. Abundance of fungal communities

The determination of fungal abundance through the number of copies of the ITS region of the ribosomal RNA gene, quantified by quantitative PCR (qPCR), reveals the distribution of fungal community representatives in the soils according to the level of HM contamination and soil depth (figure 12).

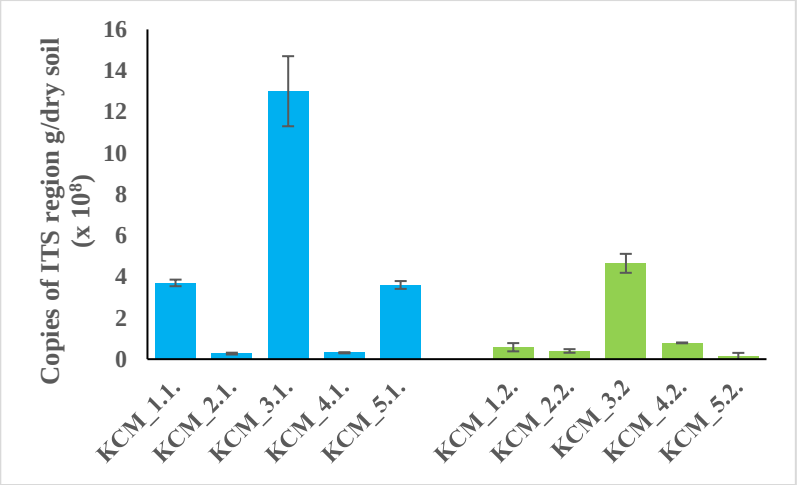


Figure 12. Abundance of fungal communities in soils expressed as the number of copies of the ITS region of the rRNA gene (per g of dry soil)

In the surface soil layer, the highest abundance of ITS region copies of the rRNA gene was observed in the slightly contaminated soil KCM_3.1 (12.6±1.69 x10^8), followed by KCM_1.1 (3.73±0.16 x10^8) and KCM_5.1 (3.55±0.19 x10^8). Lower numbers of ITS region copies were recorded in the highly contaminated soils KCM_4.1(0.31±0.03 x10^8) и KCM_2.1 (0.26±0.04 x10^8).

In the subsurface soil layer, the highest abundance of ITS region copies was again found in the slightly contaminated soil KCM_3.2 (4.65±0.46 x10^8), followed by KCM_5.2 (1.36±0.17 x10^8). Lower values were detected in the highly contaminated soils KCM_4.2 (0.78±0.02 x10^8), KCM_2.2 (0.39±0.09 x10^8) and KCM_1.2 (0.58±0.19 x10^8).

A significantly higher concentration of ITS region copies was observed in the surface layer (average 4.09±0.42 x10^8) compared to the subsurface layer (average 1.56±0.22 x10^8), indicating a 162.18% greater abundance. In soils KCM_1, KCM_3 and KCM_5, the number of ITS region copies was higher in the surface layer, whereas in KCM_2 and KCM_4, the opposite trend was observed, with higher ITS copy numbers in the subsurface layer. The distribution of ITS copies with soil depth was statistically significant ($F=6.76$; $p=0.015$).

The slightly contaminated soils from KCM_3 differed significantly in ITS copy numbers compared to all other soils ($F>6.79$; $p<0.0005$). No statistically significant differences in ITS copy number were found among the remaining soils .

4.6.2. Taxonomic composition and structure of the fungal communities

Targeted amplicon sequencing of the ITS region involves amplification of a specific segment of the internal transcribed spacer (ITS) region of the rRNA gene, followed by high-throughput sequencing.

4.6.2.1. Alpha diversity of fungal communities based on targeted amplicon sequencing of the ITS region

A total of 2889-11,045 paired-end sequences of the ITS region were obtained for the ten soil samples, which were clustered into OTUs ranging from 44 (KCM_1.1) to 118 (KCM_4.1). Based on these data, alpha diversity indices (Shannon and Simpson) were calculated. The results are presented in table 9.

The highest number of OTUs was observed in the contaminated soils KCM_2.1 (117), KCM_4.1 (118) and KCM_4.2 (107). High fungal diversity, expressed through Shannon and Simpson indices, was again found in KCM_2.1 (5.991 and 0.975, respectively). The moderately contaminated surface soil KCM_5.1 showed the lowest OTU number (32) and Shannon index (3.702). The lowest Simpson index was observed in the highly contaminated soil KCM_1.1 (0.867) (table 9).

Table 9. Characteristics and alpha diversity indices of fungal communities from surface and subsurface soils based on targeted amplicon sequencing of the ITS region.

Soil	Sequences		Alfa diversity indices	
	Reads	Total OTUs	Shannon	Simpson
KCM_1.1	5203	44	3.757	0.867
KCM_2.1	7037	117	5.991	0.975
KCM_3.1	5121	97	5.714	0.972
KCM_4.1	11045	118	4.836	0.909
KCM_5.1	2588	32	3.702	0.880
KCM_1.2	6189	60	4.072	0.863
KCM_2.2	2889	60	4.944	0.949
KCM_3.2	3594	50	4.637	0.941
KCM_4.2	6178	107	5.419	0.964
KCM_5.2	4066	47	4.274	0.908

4.6.2.2. Taxonomic composition and structure of fungal communities at the phylum level

The taxonomic composition of the fungal communities was represented by four phyla (figure 13A). The dominant phylum in both surface and subsurface soil layers was Ascomycota, accounting for 95.2-99.79% of total abundance. It was followed by Fungi_Unclassified (1.22-1.70%), which was detected in highly contaminated soils KCM_1.1 (1.7%), KCM_2.1 (1.26%) and KCM_4.2 (1.52%), as well as in the slightly contaminated soil KCM_3.2 (1.22%) at relatively low abundance. Basidiomycota was found only in KCM_3.1 (1.29%) and KCM_4.2 (1.14%) and at low abundance.

4.6.2.3. Taxonomic composition and structure of fungal communities at the class level

At the class level, ten different fungal classes were identified across the analyzed soils (figure 13B). Three of them – Sordariomycetes (average 34.49% of total abundance), Eurotiomycetes (average 34.02%), and Dothideomycetes (average 13.17%) – were almost universally distributed. Sordariomycetes and Eurotiomycetes can be considered dominant classes in the fungal communities, while the remaining classes are subdominant.

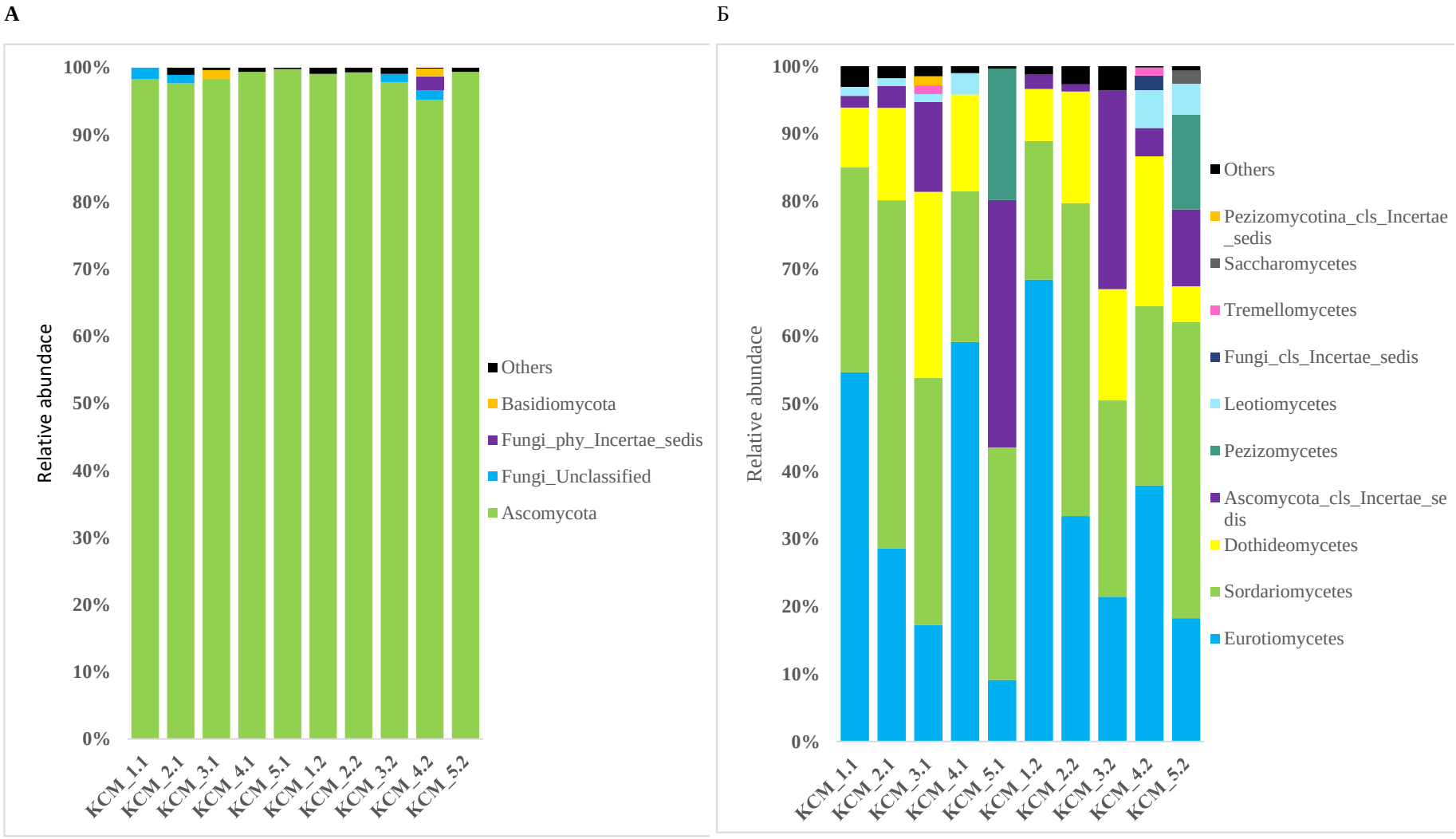


Figure 13. Relative abundance of fungal communities at the phylum level (A) and class level (B) in the soils. “Others” represent unclassified taxa with a relative abundance of <1%.

A multivariate ordination analysis was performed to determine the similarity of fungal communities based on the distribution of classes within them (figure 14).

The ordination based on the taxonomic composition of fungal communities formed two main groups. The first group includes KCM_1 and KCM_4 (highly contaminated soils; NPI>21.76), while the second group consists of soils KCM_2.1, KCM_2.2 (also highly contaminated but with NPI<9.27) and KCM_5.2 (moderately contaminated; NPI=2.5). The remaining soils (KCM_3.1, KCM_3.2 and KCM_5.1) do not form a compact group, although all are located in the fourth quadrant of the ordination plot, suggesting relatively close similarity. To examine the influence of contamination level and soil depth on the taxonomic distribution (similarity) of fungal communities, a two-way PERMANOVA was conducted (table 10).

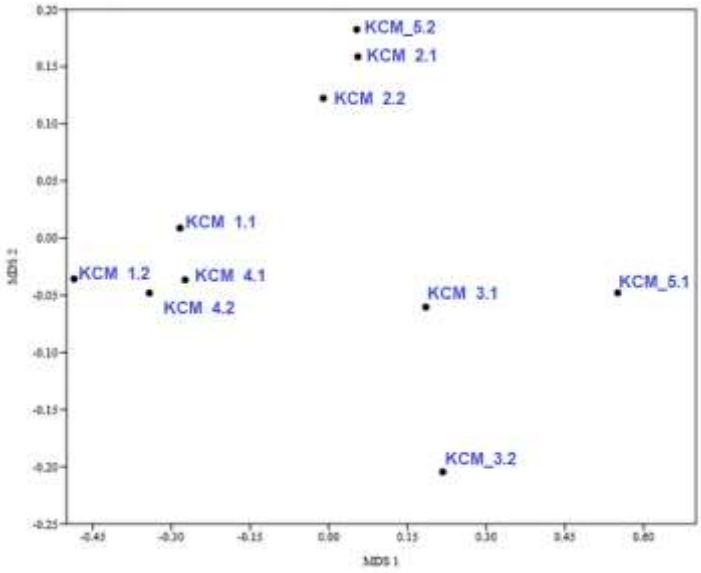


Figure 14. MDS plot of taxonomic similarity of soil fungal communities at the class level based on the Euclidean similarity index. Stress: 0.077.

Table 10. Results of two-way PERMANOVA showing the effects of heavy metal contamination level and soil depth on the distribution of fungal classes.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	p
NPI	17790.3	4	4447.6	3323.7	0.0001
Depth	85.6214	1	85.621	63.984	0.0001
Interaction (NPI×Depth)	3264.6	4	816.15	609.9	0.0001
Residuals	26.7632	20	1.3382		
Total	21167	29			

As with bacterial communities, both the level of contamination and soil depth were significant factors determining the distribution of fungal classes. The interaction between them was also significant. In contrast to bacteria, the interaction between contamination and depth (F=609; p=0.0001) had a greater effect than depth alone (F=63.98; p=0.0001). No significant correlations were found between soil depth and fungal

classes, which is consistent with the PERMANOVA results indicating the relatively weak influence of soil depth on fungal distribution.

To identify the classes contributing most to between-group dissimilarities among fungal communities, a SIMPER analysis was performed (table 11).

Table 11. Results of SIMPER analysis showing the contribution of taxonomic classes to the total dissimilarity (37.18%) among soil fungal communities.

Source of variation	Average dissimilarity	Individual contribution (%)	Cumulative contribution (%)
Eurotiomycetes	12.64	34	34
Ascomycota_cls_Incertae_sedis	7.243	19.48	53.48
Sordariomycetes	6.837	18.39	71.87
Dothideomycetes	5.145	13.84	85.71
Pezizomycetes	3.392	9.124	94.84
Leotiomycetes	1.154	3.104	97.94
Tremellomycetes	0.2193	0.5899	98.53
Fungi_cls_Incertae_sedis	0.2058	0.5535	99.08
Saccharomycetes	0.2012	0.5411	99.63
Pezizomycotina_cls_Incertae_sedis	0.1393	0.3748	100

The greatest contribution (34%) to the total dissimilarity among fungal communities was attributed to variability in the abundance of the class Eurotiomycetes. This class showed a significant and positive correlation with the level of contamination.

4.6.2.4. Taxonomic composition and structure of fungal communities at the genus level

The taxonomic profiling of fungal communities at the genus level revealed remarkable richness, with a total of 57 distinct genera identified (figure 15). The greatest diversity was observed in the subsurface layers of the highly contaminated soils KCM_2.2 and KCM_4.2 (20 genera each), while the lowest diversity was recorded in both the surface and subsurface layers of the moderately contaminated soil KCM_5 (7 genera in KCM_5.1 and 10 genera in KCM_5.2).

One of the dominant genera in soils highly contaminated with HMs was *Bacillicladium*. Its relative abundance was highest in KCM_1.1 (48.37%), KCM_1.2 (55%), KCM_4.1 (42.63%) and KCM_4.2 (19.1%), while in KCM_2.1 and KCM_2.2 its proportion decreased to 4.91% and 3.64%, respectively. Another dominant genus in these soils was *Fusarium*, with relative abundances of 22.67% in KCM_1.1, 14.34% in KCM_2.1 and 13.72% in KCM_4.2. It is also noteworthy that *Penicillium* was detected in the highly contaminated soils KCM_4.1 (8.21%) and KCM_4.2 (5.43%), although in lower relative abundance. *Neoschizothecium* was found to dominate exclusively in KCM_2.2 (22.67%) and KCM_2.1 (18.14%).

In the moderately contaminated soils KCM_5.1 and KCM_5.2, the genus *Enterocarpus* was characteristic, comprising 27.64% and 36.32% of the fungal communities, respectively. In addition to this genus, *Cephaliophora* (4.36-7.74%) and *Coniochaeta* (1.22-3.95%) were also detected, though at lower proportions.

In the slightly contaminated soils, the fungal communities exhibited the highest genus-level diversity in the surface layer of KCM_3.1, where 18 genera were identified, compared to only 11 genera in

the subsurface layer of KCM_3.2. These soils also contained a significant proportion of unidentified genera labeled as "Others", representing 30% of the fungal community in the surface layer and 12.89% in the subsurface layer.

Among the dominant genera in the slightly contaminated soils were *Fusarium* (13.73-15.64%) and *Chrysosporium* (5.64-8.83%). The genera *Sirastachys* (1.41-1.72%) and *Trichoderma* (1.27-.47%) were also characteristic of these soils, occurring at both depths, though in low relative abundance.

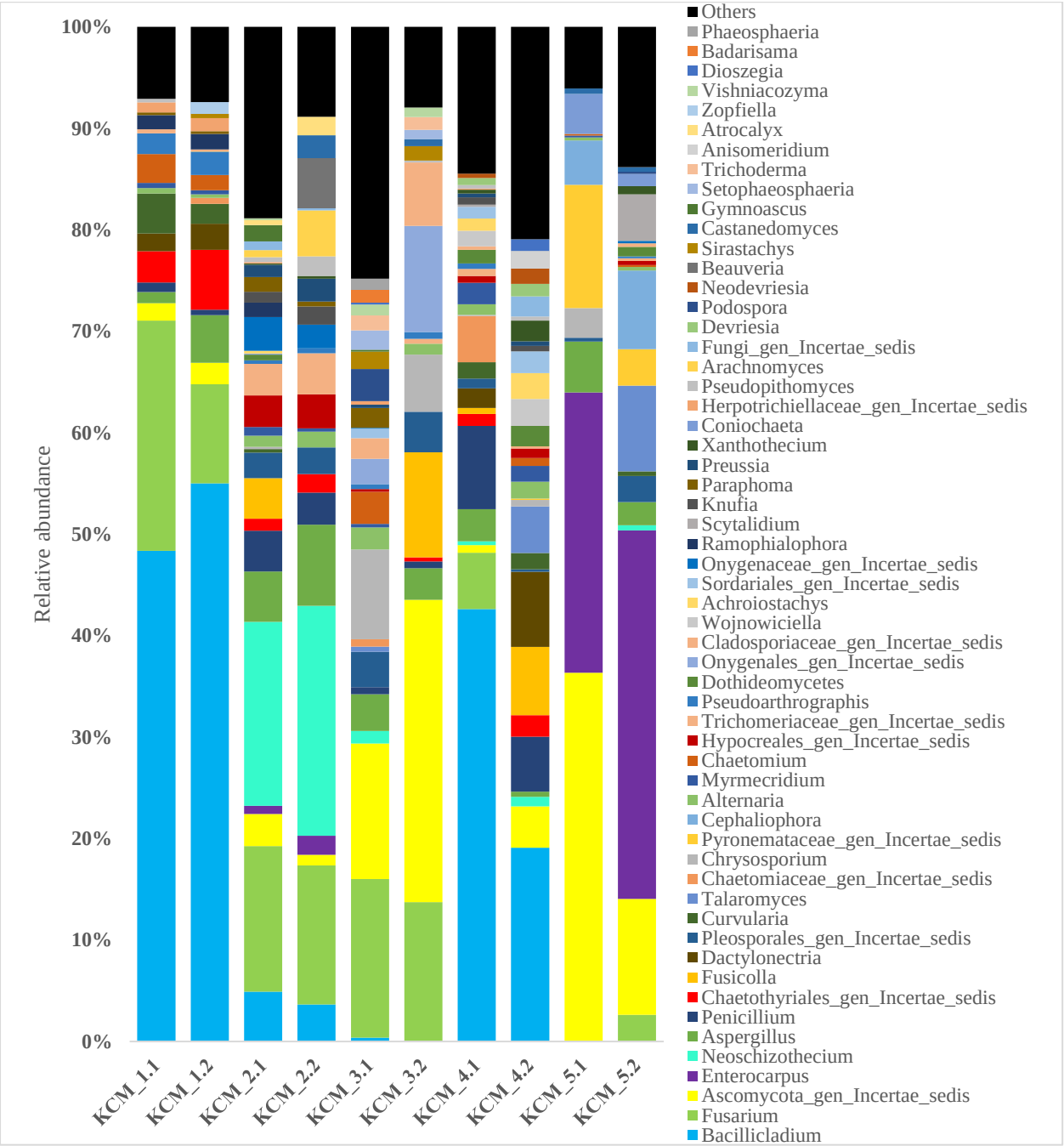


Figure 15. Relative abundance of fungal communities at the genus level in the studied soils. "Others" represent unclassified taxa with relative abundance <1%

4.7. Influence of environmental factors on the biological diversity of microbial communities

This section examines the potential effects of the studied environmental factors on the taxonomic composition of bacterial and fungal communities. The impact was assessed at the genus level, as microbial responses at this taxonomic rank are considered to be more specific and informative than those observed at higher taxonomic levels. To evaluate these relationships, a CCA was performed. The distribution of the soils according to the composition of the bacterial (A) and fungal (B) communities is illustrated in figure 16.

The first two ordination axes together explained 57.54% (for bacterial communities) and 50.70% (for fungal communities) of the total variance in community structure. The ordination plot clearly shows grouping of the communities according to the level of HM contamination. For both domains, the slightly contaminated soil KCM_3 and the moderately contaminated soil KCM_5 are positioned on the left side of the diagram – KCM_3 in the upper left quadrant and KCM_5 in the lower left quadrant. The communities of the highly contaminated soils are located on the right side of the diagram. Among the bacterial communities, this group is more scattered, whereas among the fungal communities KCM_2 (NPI: 7.99-9.27) occupies the upper right quadrant and KCM_1 and KCM_4 (NPI: 21.76-67.43) are positioned in the lower right quadrant. Each group of soils is associated with the predominant presence of certain characteristic bacterial and fungal genera. For example, the bacterial communities of KCM_3 are characterized by the presence of genera *AC-51* (B27; UBA4738_401450) and *HRBIN40* (B29; Alphaproteobacteria), which are considered sensitive to HMs. In contrast, the highly contaminated soils are characterized by genera such as *UBA2421* (B2), *AG11* (B9), *Aquicella_A* (B32; Gammaproteobacteria), *ELB16-189* (B38; Bacteroidia), and *Z2-YC6860* (B39; Alphaproteobacteria) – notably present in KCM_1 and KCM_4.1. In the fungal communities, the genera characteristic of KCM_3 includes *Aspergillus* (F6; Eurotiomycetes), *Chrysosporium* (F15; Eurotiomycetes), *Podospora* (F43; Sordariomycetes), *Neodevriesia* (F44; Sordariomycetes), among others. Conversely, the genera typical of highly contaminated soils (KCM_1 and KCM_4) include *Bacillicladium* (F1; Eurotiomycetes), *Dactylonectria* (F10; Sordariomycetes), and *Pseudoarthrographis* (F23; Dothideomycetes), among others. Representatives of the first group of genera are regarded as low-tolerant to HM stress, whereas those of the second group are considered resistant to its effects.

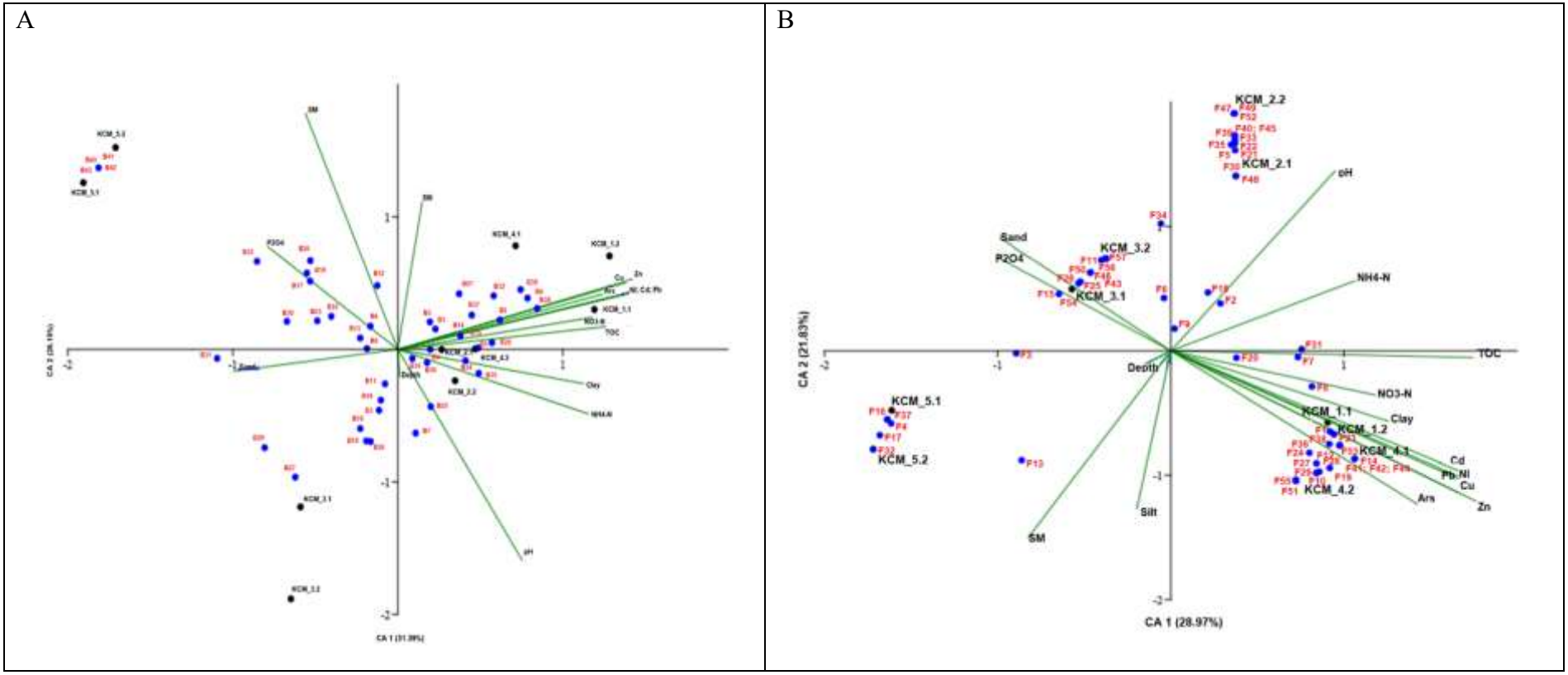


Figure 16. CCA plot illustrating the influence of major environmental factors (vectors) on the distribution of bacterial (A) and fungal (B) genera (marked in red) within the soil microbial communities from the KCM 2000 area.

The presence of these genera in the highly contaminated soils indicates a well-developed adaptive strategy, high resistance and the ability to exploit the advantages of the habitat. A positive correlation exists between all of the above-listed bacterial and fungal genera and the soil concentrations of HMs, as well as the overall pollution level. It can be assumed that the concentration of HMs is the main determining factor for their distribution.

For some genera, the influence of environmental factors is complex; besides the impact of HMs, other factors also affect their distribution. For example, in the bacterial genera *Gp6-AA45* (B1; Vicinamibacteria), *Sulfotelmatobacter* (B15; Acidobacteriae) and *Haliangium*_463188 (B28; Polyangia_463783), not only HMs but also TOC and NO_3-N have a positive effect, while a higher sand fraction exerts a negative influence. Bacterial genera influenced both by HMs and by higher clay content and NH_4-N include *Pedosphaera* (B24; Verrucomicrobiae) and *Blastococcus* (B35; Actinomycetia).

The phosphate content of the soil has a strong effect on the distribution of many bacterial genera, including *Devosia_A_502124* (B40; Alphaproteobacteria), *Luteimonas_C_615545* (B41; Gammaproteobacteria), *Singulisphaera* (B42; Planctomycetia), and *Corynebacterium* (B43; Actinomycetia). Soil pH affects the distribution of *Solirubrobacter* (B7; Gemmatimonadetes) and *Chryseolinea* (B25; Bacteroidia), whereas the occurrence of *SCUD01* (B31; Gammaproteobacteria) is influenced by the proportion of sand particles.

The distribution of many HM sensitive fungal genera (F15, F25, F26, F43, F50, F54, etc.) is associated not only with their sensitivity but also with increased soil phosphate levels and changes in soil texture toward a more sandy composition. Many fungal genera are affected by pH (F2, F18, F48), total organic carbon (F7, F20, F31), or nitrate nitrogen (F8). For some genera (F13), soil moisture is a critical factor.

Overall, it can be concluded that the distribution of microbial genera in the soil is primarily determined by their sensitivity or resistance to HM concentrations, as well as by phosphate and organic carbon content, soil pH, and texture. Unlike PERMANOVA, the CCA did not identify a substantial effect of soil depth on the distribution of bacterial and fungal genera (PERMANOVA indicated a weak, though statistically significant, influence).

4.8. Metabolic functions of microbial communities

4.8.1. Predicted functional profiles of bacterial communities based on metagenomic sequencing

Metagenomic analysis allows the prediction of metabolic profiles of bacterial communities based on the sequences obtained from targeted amplicon sequencing of the 16S rRNA gene. This prediction was performed using the PICRUSt2 software, which relies on the KEGG Orthology (KO) database.

The predicted functions of the bacterial communities, as determined by PICRUSt2, revealed the presence of various KEGG pathways, ranging from 5389 (KCM_3.2) to 6199 (KCM_5.1), with frequency between 16604969 (KCM_3.2) and 22202383 (KCM_2.1) (table 12).

Table 12. Number and frequency of predicted KEGG pathways in the soils.

Soil	Number of KEGG pathways	Frequency
KCM_1.1	5693	19410771
KCM_2.1	6074	22202383

KCM_3.1	5702	21819060
KCM_4.1	5537	18388859
KCM_5.1	6199	18893739
KCM_1.2	5400	20959375
KCM_2.2	5774	17621695
KCM_3.2	5389	16604969
KCM_4.2	5555	19114849
KCM_5.2	6046	17953730

Of particular interest is the identification of metabolic pathways, enzymes, or proteins involved in bacterial adaptation to HM-contaminated environments. The targeted analysis revealed 26 enzymes and proteins with varying sequence abundances across all studied soils. These 26 enzymes and proteins were grouped into two main clusters and several subclusters (figure 17).

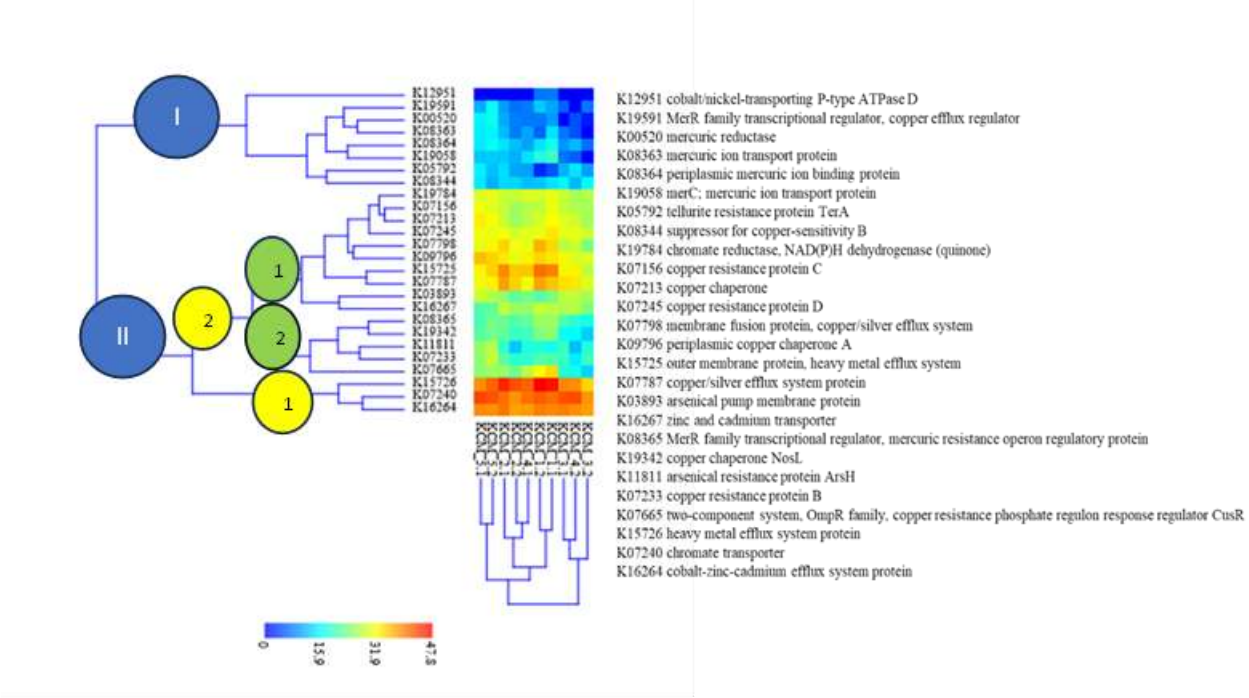


Figure 17. Dendrogram based on Box–Cox transformed data matrix of KEGG metabolic pathways associated with the adaptation of soil bacteria to HM contaminated environments.

The analysis identified two main clusters (marked in blue in figure 17).

Cluster I includes proteins and enzymes with the lowest abundance (0-884) in the soils, which play a role in HM detoxification, mainly of Hg. These are:

1. cobalt/nickel-transporting P-type ATPase D ctpD (K12951) – transporter for cobalt and nickel. Regulates intracellular levels of cobalt and nickel, preventing toxicity.
2. MerR family transcriptional regulator, copper efflux regulator cueR (K19591) – transcriptional regulator of the copper efflux system; controls the expression of genes related to copper resistance.
3. mercuric reductase merA (K00520) – enzyme that reduces mercuric ions to elemental mercury, aiding in cellular detoxification.

4. mercuric ion transport protein merT (K08363) – protein responsible for the transport of mercuric ions; part of the mercury detoxification mechanism.
5. periplasmic mercuric ion binding protein merP (K08364) – binds mercuric ions in the periplasmic space; contributes to detoxification.
6. mercuric ion transport protein merC (K19058) – protein facilitating transport of mercuric ions; aids mercury detoxification.
7. tellurite resistance protein TerA (K05792) – protein conferring resistance to tellurite; protects bacterial cells from tellurite toxicity.
8. suppressor for copper-sensitivity B scsB (K08344) – protein that suppresses copper sensitivity.

Cluster II is divided into two subclusters (marked in yellow in figure 17). Subcluster 1 includes the three most abundant proteins (abundance 5238-15794) involved in efflux systems that protect cells from HM toxicity: These are:

1. heavy metal efflux system protein czcA, cusA, cnrA (K15726) – participates in detoxification of Co^{2+}, Zn^{2+}, and Cd^{2+} by pumping them out of the cell.
2. chromate transporter ChrA (K07240) – component of efflux systems responsible for chromium removal from the cell.
3. cobalt-zinc-cadmium efflux system protein (K16264) – protects bacteria from toxic concentrations of Co^{2+}, Zn^{2+}, and Cd^{2+} by exporting them from the cell.

Subcluster 2 contains two well-defined subdivisions (marked in green):

1) Proteins and enzymes with high abundance (1068-9346) responsible for reducing the toxicity of HMs (mainly Cu, Zn, Cd, Cr and As) within the cell:

1. chromate reductase, NAD(P)H dehydrogenase (quinone) (K19784) – enzyme that reduces chromate to less toxic forms.
2. copper resistance protein C, copC, pcoC (K07156) – protects cells from toxic copper concentrations.
3. copper chaperone ATOX1, ATX1, copZ, golB (K07213) – ensures proper intracellular transport and distribution of copper.
4. copper resistance protein D pcoD (K07245) – contributes to copper detoxification.
5. membrane fusion protein, copper/silver efflux system cusB, silB (K07798) – component of the efflux system for copper and silver; participates in metal export mechanisms.
6. periplasmic copper chaperone A pccA (K09796) – periplasmic copper chaperone aiding in proper distribution and detoxification of copper.
7. outer membrane protein, heavy metal efflux system czcC K15725 – part of the efflux system responsible for the removal of Co^{2+}, Zn^{2+} and Cd^{2+}.
8. copper/silver efflux system protein cusA, silA (K07787) – component of the copper and silver efflux system.
9. arsenical pump membrane protein, arsB (K03893) – membrane protein that exports arsenic from the cell.
10. zinc and cadmium transporter (K16267) – regulates intracellular Zn and Cd levels by exporting excess metals, preventing toxicity.

2) Proteins and enzymes (abundance: 205–5,073) associated with cellular resistance to copper and arsenic, including:

1. MerR family transcriptional regulator, mercuric resistance operon regulatory protein (K08365) – regulates expression of genes associated with mercury resistance.
2. copper chaperone NosL (K19342) – facilitates copper transport and distribution within the cell.
3. arsenical resistance protein ArsH (K11811) – confers resistance to arsenic.
4. copper resistance protein B pcoB, copB (K07233) – involved in copper resistance.
5. two-component system, OmpR family, copper resistance phosphate regulon response regulator CusR (K07665) – regulatory protein of a two-component system controlling copper resistance genes.

Overall, cluster I encompasses defense mechanisms primarily against Hg, whereas cluster II includes systems predominantly involved in cell protection from Cu, Zn, Cd, and As. The frequency of detoxification systems for Cu, Zn, Cd, and As is much higher than those for Hg, which corresponds to the observed contamination profile.

The results of the metagenomic analysis were also used to predict existing KEGG enzymes (pathways) associated with energy metabolism and regulation of cellular biochemical processes in bacteria. The most frequently detected enzymes were:

1. 3-oxoacyl-[acyl-carrier protein] reductase (K00059) - involved in fatty acid biosynthesis.
2. acetyl-CoA C-acetyltransferase (K00626) - participates in lipid metabolism.
3. UDP-glucose 4-epimerase (K01784) - involved in carbohydrate metabolism.
4. long-chain acyl-CoA synthetase (K01897) - activates long-chain fatty acids.
5. serine/threonine protein kinase, bacterial (K08884) - participates in signal transduction through phosphorylation of serine/threonine residues, regulating enzyme and protein activity during cellular and stress responses.

The frequency of these enzymes in the studied soils ranged from 24358 (KCM_5.2) to 79110 (KCM_3.1).

Additionally, KEGG enzymes involved in the degradation of organic matter were grouped according to the type of biochemical reactions and degradation mechanisms:

- hydrolases (amylases or carbohydrases, proteases, lipases, deaminases and decarboxylases);
- oxidoreductases (dehydrogenases).

The distribution of these enzyme groups across the two soil depths, expressed as mean occurrence frequency, is shown in figure 18.

The oxidoreductases showed the highest mean frequency (surface soil – 32778; subsurface soil – 30462), followed by deaminases/decarboxylases (surface – 13356; subsurface – 11870) and proteases (surface – 9762; subsurface – 8517). Lipases exhibited the lowest occurrence (surface – 3385; subsurface – 3140). Overall, all enzyme groups were more frequent in the surface than in the subsurface layer, although the difference was not statistically significant ($F<2.92$; $p>0.12$).

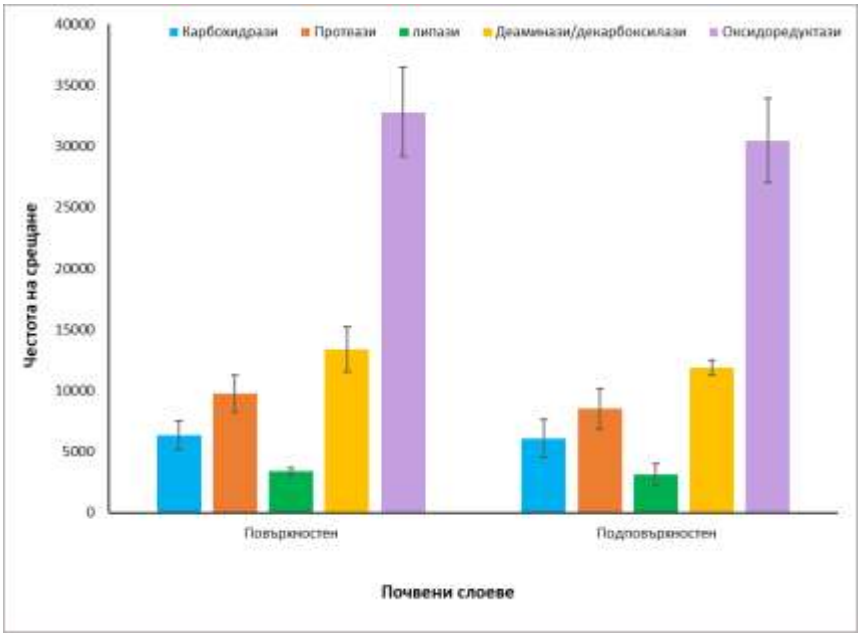


Figure 18. Frequency of occurrence of enzymes belonging to the groups of hydrolases and oxidoreductases in the two soil depths..

4.8.2. Metabolic potential and metabolic profiles of soil bacterial communities determined by the Biolog Ecoplate™ system

To gain deeper insight into the influence of pollution and soil depth on bacterial metabolism, the Biolog EcoPlate™ system was used. This system reflects the metabolic characteristics of culturable heterotrophic bacteria. The main parameters analyzed were: (1) average metabolic activity (AWCD); (2) community-level physiological profiles (CLPP); and (3) functional diversity (Shannon index) of the heterotrophic bacterial communities.

4.8.2.1. Average metabolic activity (AWCD)

The average well color development (AWCD) serves as an indicator for assessing the ability of soil bacterial communities to metabolize a set of 31 natural carbon sources included in the EcoPlate microtiter plate. AWCD values varied depending on the sampling site and the metabolic activity results are presented in figure 19. The potential of bacterial communities from the KCM 2000 area ranged from 2.04±0.042 AUC (KCM_1.1) to 7.79±0.146 AUC (KCM_2.1).

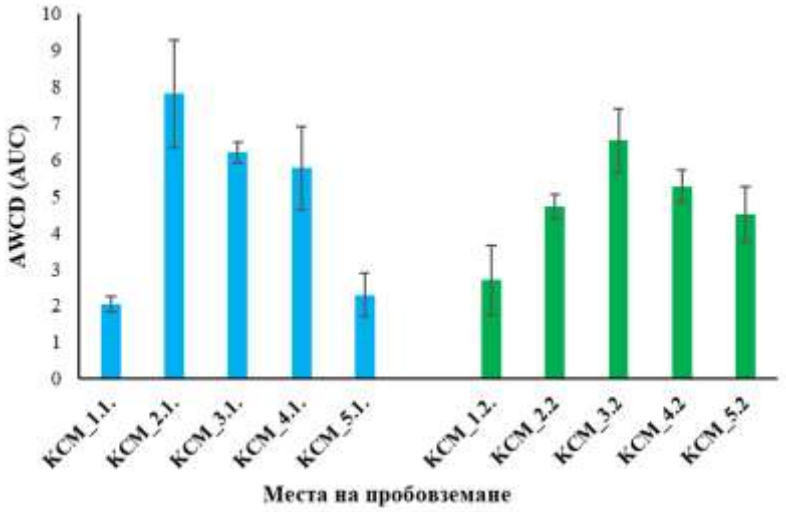


Figure 19. Metabolic potential of bacterial communities from surface and subsurface soil layers to utilize carbon sources.

Bacterial communities from KCM_1.1 (2.04±0.017 AUC), KCM_1.2 (2.69±0.96 AUC), and KCM_5.1 (2.29±0.96 AUC) were characterized by low metabolic potential. The likely reason for this is the unfavorable environmental conditions, mainly associated with the high levels of HM contamination in the soils (KCM_1). In KCM_5.1, the contamination is considered moderate; however, other specific environmental factors may also suppress the metabolic activity of heterotrophic bacterial communities.

A higher metabolic potential was observed in the bacterial communities from the control soil KCM_3 (average 6.34±0.61 AUC), which is the least contaminated with HMs, and from KCM_2.1 (7.79±1.46 AUC), characterized by higher total organic carbon content (14.07 g/kg) and lower contamination level (NPI: 7.99).

The mean AWCD value of the surface soil layer (4.81±2.54 AUC) was very close to that of the subsurface layer (4.74±1.39 AUC) and the difference was not statistically significant (F=0.01; p=0.92).

4.8.2.2. Functional profile of bacterial communities (CLPP)

CLPP was determined based on the level of utilization of biochemical groups of carbon sources as well as individual substrates. The carbon sources in the EcoPlate microtiter plates were grouped into five biochemical categories – carbohydrates (CH), polymers (Polym), carboxylic acids (CA), amino acids (AA), and amines/amides (Amin) (Weber and Legge, 2009). The contribution of these biochemical groups to the total metabolic activity of the soil bacterial communities is presented in figure 20.

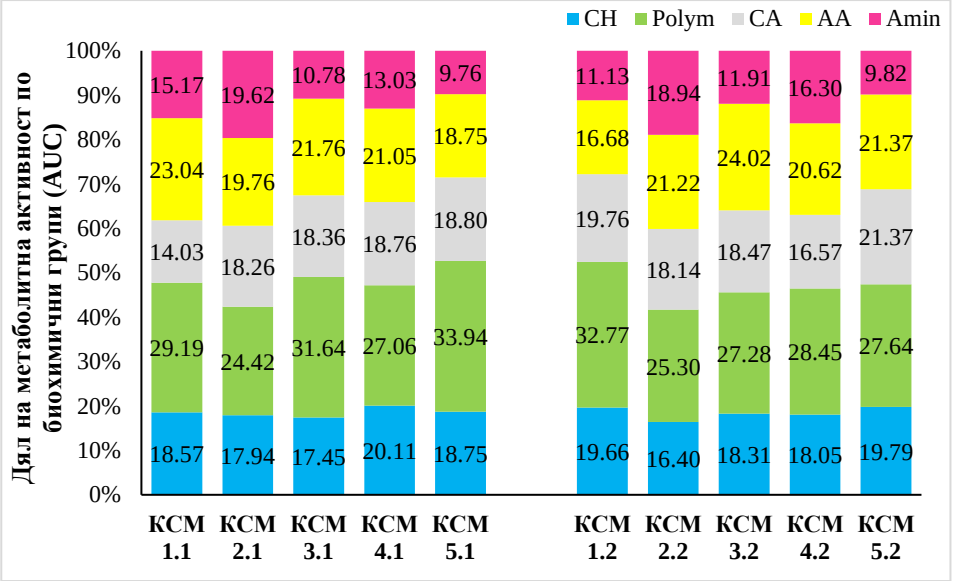


Figure 20. Potential of soil bacterial communities to utilize different biochemical groups of carbon containing compounds as a percentage of total metabolic activity.

The results demonstrate a tendency toward relatively uniform utilization of the different biochemical groups of carbon containing compounds across all soils. The most highly utilized group was Polym, accounting for 24.42-33.94% of the AWCD, followed by CH (16.4-20.11%) and protein- (AA) and non-protein-forming carboxylic acids (CA) (14.03-24.02%). The least utilized group was Amin (9.76-19.62%). There were minor deviations from this pattern; for example, in soils KCM_2 and KCM_1.1, the least utilized groups were CH and CA, respectively, rather than Amin.

4.8.2.3. Functional diversity

The CLPP data were used to calculate the following indices:

- functional diversity (Shannon index, H′);
- evenness of carbon source utilization (Pielou index, E).

The results are presented in table 13.

Table 13. Functional diversity indices of soil bacterial communities from surface and subsurface soil layers.

Soil	Shannon (H′)	Pielou (E)
КСМ 1.1	3.284±0.14	0.956±0.02
КСМ 2.1	3.342±0.05	0.973±0.02
КСМ 3.1	3.335±0.06	0.971±0.02
КСМ 4.1	3.343±0.06	0.973±0.02
КСМ 5.1	3.291±0.12	0.958±0.02
КСМ 1.2	3.289±0.11	0.958±0.02
КСМ 2.2	3.302±0.08	0.962±0.01
КСМ 3.2	3.283±0.06	0.969±0.02
КСМ 4.2	3.336±0.06	0.972±0.02
КСМ 5.2	3.350±0.07	0.976±0.01

The calculated indices indicate a high functional diversity and a high degree of evenness in the utilization of carbon sources in the EcoPlate system. No statistically significant differences were observed between the soils ($F<0.29$, $p>0.88$) or between the soil depths ($F<0.006$, $p>0.92$) for these indices. These results demonstrate a preserved broad-spectrum metabolic activity even under conditions of high HM

contamination. This finding is not unexpected, given the long-term nature of the pollution (more than two decades) and the likely realized adaptive potential of bacteria to thrive in their contaminated habitat.

4.8.3. Soil enzyme activities

The activities of six soil enzymes were determined – total dehydrogenase (Dha), β-glucosidase (BGl), urease (Ur), acid phosphatase (AcP), alkaline phosphatase (AlP) and arylsulfatase (Ars) – in order to assess the influence of HMs on them and, consequently, on the biogeochemical cycles in which these enzymes participate.

The trend of variation in the overall enzyme activity index is shown in figure 21, while the measured enzyme activity values are presented in table 14.

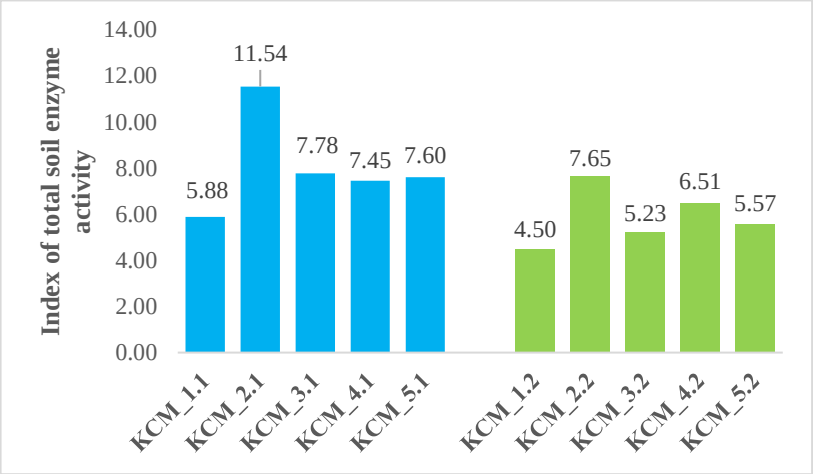


Figure 21. Index of total soil enzyme activity..

The lowest overall enzyme activity index was observed in KCM_1.2 (4.50), while the highest occurred in KCM_2.1 (11.54). Among surface soils, KCM_1.1 (5.88) also showed a low enzyme activity index. The total enzyme activity varied significantly depending on the sampling site ($F=24.98$, $p<0.001$). A significant difference was also found between the two soil depths ($F=12.3$, $p=0.002$). In the surface soil layer, enzyme activities were generally higher than those in the subsurface layer, with the exception of urease (Ur), whose activity did not depend on soil depth ($F=0.003$; $p=0.95$). Specifically, in the surface layer, enzyme activities were higher by 172% (Dha), 54% (BGl), 39% (AcP), 32% (AlP) and 7% (Ars) compared to those in the subsurface layer.

The lowest activities of Dha, AlP, and Ars were recorded in KCM_1.2, while BGl and AcP showed their lowest values in KCM_5.2.

Table 14. Enzyme activity values for Dha, BGl, AlP, AcP, Ars, and Ur (μg/g/h).

Soil	Dha	BGl	AlP	AcP	Ars	Ur
KCM_1.1	24.21 ± 0.4	20.82 ± 6.7	18.06 ± 2.4	8.27 ± 2.5	2.57 ± 0.2	1.68 ± 0.1
KCM_2.1	60.91 ± 7.4	61.71 ± 6.6	49.51 ± 7.6	10.02 ± 1.0	6.82 ± 0.9	2.26 ± 0.2
KCM_3.1	40.32 ± 6.1	19.68 ± 6.5	21.22 ± 7.6	8.72 ± 1.5	6.98 ± 1.7	2.14 ± 0.2
KCM_4.1	45.27 ± 6.2	23.59 ± 4.3	23.30 ± 3.2	13.04 ± 2.1	2.50 ± 0.6	1.86 ± 0.6
KCM_5.1	31.96 ± 5.6	14.73 ± 6.8	39.25 ± 0.3	8.19 ± 0.6	2.24 ± 0.8	3.45 ± 1.0

KCM_1.2	7.10 ± 3.2	28.57 ± 3.4	14.53 ± 1.3	5.48 ± 0.3	1.61 ± 0.5	1.62 ± 0.3
KCM_2.2	20.32 ± 7.4	33.21 ± 4.3	36.29 ± 7.4	6.60 ± 0.8	5.08 ± 0.8	2.04 ± 0.4
KCM_3.2	11.95 ± 0.1	8.78 ± 0.6	18.71 ± 0.8	7.90 ± 0.9	5.12 ± 0.6	1.40 ± 0.5
KCM_4.2	16.84 ± 3.3	16.85 ± 3.9	27.26 ± 1.3	9.49 ± 1.6	2.36 ± 1.1	2.40 ± 0.7
KCM_5.2	14.05 ± 3.4	8.25 ± 0.8	23.64 ± 7.5	5.17 ± 0.5	3.22 ± 1.6	4.92 ± 0.9

The association of higher enzyme activities with less contaminated surface soils suggests that both pollution level and soil depth exert an inhibitory effect on the biological activity of soil microbial communities.

4.9. Influence of environmental factors on the functions of soil microbial communities

The effect of the analyzed abiotic environmental factors on the utilization of Biolog Ecoplate™ carbon sources and on soil enzyme activities was evaluated. For this purpose, CCA ordination technique was applied, and the results are presented in figure 22.

The ordination was based on the first two canonical axes, which together explained 62.45% of the variation in the relationship between bacterial function and environmental conditions. The first axis accounted for 45.5% of this variation and correlated mainly with soil moisture, nitrate nitrogen, total organic carbon, and HMs. The second axis, explaining 16.9% of the variation, was primarily correlated with ammonium nitrogen content.

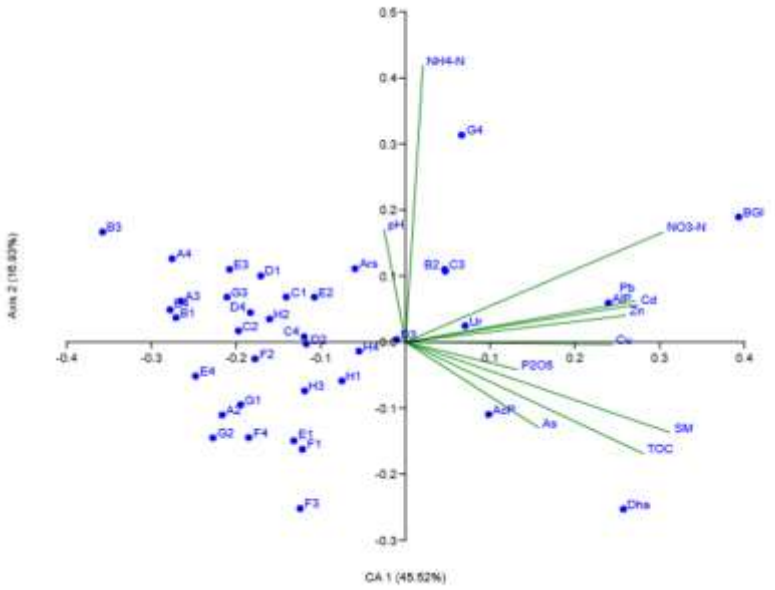


Figure 22. CCA of bacterial functional profiles, soil enzyme activities, and abiotic environmental factors illustrating the relationship between "microbial functionality – habitat environment."

The distribution of BGl, Dha and AlP enzyme activities showed a positive correlation with soil moisture, nitrate nitrogen, organic carbon, and HM content. These factors, however, exhibited a negative effect on the utilization of certain carbohydrates (B1, G1, A2, C2 and G2), protein-forming carboxylic acids (A4, B4 and E4), and non-protein-forming carboxylic acids (A3, B3, E3 and G3) (https://www.biolog.com/wp-content/uploads/2023/08/00A-012-Rev-F-EcoPlate-IFU.pdf). Additionally, the ammonium ion content in soils showed a positive influence on the metabolism of G4 (amine) and BGl activity, but a negative effect on Dha activity and on the utilization of F3 (carboxylic acid).

5. Discussion

The results obtained in the dissertation show how the specific conditions in long-term HM contaminated soils in the area of KCM 2000 - Plovdiv affect the taxonomic composition and function of the soil microbiome. With over 70 years of ore extraction and processing, the "KCM 2000" holding is a significant industrial factor with a long-term impact on the environment in the region.

5.1. Environmental characteristics

The study of environmental factors is essential for determining the effects of pollutants on the soil microbiome.

The main factor monitored in this study is the type and level of HM contamination of the soils in the KCM 2000 - Plovdiv area. A high level of contamination with Zn, Pb, Cu, Cd and As was found, with the levels of these HMs in most soils exceeding the maximum permissible concentrations (MPC) (table 1). In this case, there is combined contamination with HMs, which has different toxic effects on microbial communities. Metals such as Pb, Cd and As are characterized by a high degree of toxicity, since they have a lipophilic nature and relatively easily penetrate the cell membrane (Davidova et al., 2024; Jaishankar et al., 2014). Their content is highest in KCM_1, along with that of other contaminants.

Many authors consider that the biologically available forms of HMs are more indicative of their toxicity to soil organisms (Yang et al., 2022). In this study, these forms represent on average from 0.06% to 6.78% of the total HM content, and according to other authors (Zhu et al., 2023; Prokop et al., 2003), their concentration is not only a function of the total amount of HMs, but also depends on environmental factors such as pH, soil texture, moisture, etc. The soil KCM_1 has the highest level of contamination with biologically available forms of HMs.

Since the contamination with HMs is complex, it is important to determine a single indicator that can serve as a reference point for evaluating the impact of contamination on soil microbial communities. In this case, the Nemerow pollution index (NPI) was chosen, as it takes into account not only the concentrations of pollutants but also their individual toxicity to cells. According to NPI, KCM_3 is considered slightly contaminated, KCM_5 moderately contaminated, and KCM_2 < KCM_4 < KCM_1 represent increasingly contaminated soils with higher pollution. The ordination analysis of soils based on physicochemical characteristics clearly showed that their grouping depends on the HM content. A well-defined pollution gradient was identified and it is expected that along this gradient we will observe changes in the composition and functions of microbial communities, unless the effects of HMs are modified by specific environmental factors.

It was noted that many environmental characteristics can modify the action of HMs on soil microbial communities. The studied soils have a sandy-clay texture, which suggests a lower buffering capacity (Chen et al., 2019) and, consequently, a more pronounced biological effect of HMs. The sandy nature of the soils may also affect the diffusion of HMs (Chen et al., 2019), moisture retention and thus the physiological condition of microbial communities (Islam et al., 2022). Although soil moisture is an important indicator of both HM dynamics and microbial community condition, it is highly variable, depends on climatic conditions and provides only a snapshot of this parameter. At the time of sampling, soil moisture ranged from 6.7% to 16.7%, with slightly higher values in KCM_5 (22.7%). These moisture levels are considered sufficient to maintain a good physiological status of soil microbial communities (Siebielec et al., 2020). Similar to moisture, soil pH is an important factor for the functioning of soil organisms and the

dissociation of HMs (Sintorini et al., 2021). The pH values of the soils in the KCM 2000 area vary within a narrow range (6.6-7.2) and are close to neutral. This does not lead to significant effects on HM mobility, which is consistent with the results of Rutgers et al. (2016).

Soil organic matter, together with clay particles, determines the buffering capacity of the soil and its ability to reduce HM toxicity on microbial communities (Stefanowicz et al., 2020; Kwiatkowska-Malina, 2018). In the present study, it was found that the slightly contaminated soils KCM_3.1 and KCM_3.2 are characterized by lower TOC content (6.45 g/kg and 7.5 g/kg, respectively) compared to the average value (10.41±3.4 g/kg). It is assumed that this may be the result of higher degradative activity of heterotrophic bacterial communities compared to those in more contaminated soils. If this assumption is correct, it would mean that the HM contamination levels in soil KCM_3 are fully or partially tolerable for microbial communities. On the other hand, the higher TOC levels in more contaminated soils increase their buffering capacity and have a protective effect on soil communities.

For the stability and resilience of microbial communities, in addition to organic carbon, inorganic nitrogen and phosphorus are also important (Ogunsola et al., 2020). The contents of nitrate and ammonium nitrogen were highest in KCM_1, and that of phosphates in KCM_2. These are considered specific environmental characteristics, and it is possible that the higher level of NO_3-N in KCM_1 is also due to fertilization practices. On the other hand, microbial activity itself can affect the levels of mineral nutrients. For example, soil KCM_3.2, with the lowest moisture content (6.7%), is characterized by low P_2O_5 (4.18 mg/kg) and total mineral nitrogen (10.13 mg/g) values. The obtained results are consistent with data from the literature, which indicate that low soil moisture limits the uptake of carbon and other nutrients by bacterial communities, thereby reducing their enzymatic activity and potential for organic matter formation (Butcher et al., 2020).

5.2. Abundance and diversity of soil microbial communities

The abundance and diversity of bacterial communities were determined using the most commonly applied indicators (CFU, copies of the 16S rRNA gene, 16S rRNA gene clone libraries) and indices (Chao1, ACE, Shannon). The obtained results are not unidirectional, which, in our opinion, is due to the technique used. When cultivation methods, quantitative PCR (qPCR), and the construction of 16S rRNA gene clone libraries were applied, the highest abundance was observed in slightly to moderately contaminated soils KCM_3.1 (CFU, 16S rRNA gene copies, and 16S rDNA) and KCM_5.1 (16S rRNA gene copies and 16S rDNA), but high abundance was also reported in the highly contaminated soils KCM_2.2 (16S rRNA gene copies and 16S rDNA) and KCM_1.2 (16S rDNA). The obtained results are consistent with our previous studies, in which bacterial abundance (CFU and 16S rRNA gene copies) decreased in long-term Cu-, Zn-, and Pb-contaminated soils from the Zlatitsa-Pirdop area (Aleksova et al., 2020; Palov et al., 2020). Our results are also in agreement with the general trend in the scientific literature, according to which the number of cultivable heterotrophic bacteria decreases with increasing HM concentrations (Pacwa-Plociniczak et al., 2018). Other authors report the opposite trend, that HMs (Cu, Zn, Pb and Cd) have little effect on abundance but a strong effect on the diversity of bacterial communities (Stan et al., 2011; Tipayno et al., 2018; Huang et al., 2021).

Bacterial diversity determined by 16S rDNA libraries was highest in KCM_5.1, KCM_5.2 and KCM_3.1 (slightly to moderately contaminated soils) and lowest in KCM_1.1 and KCM_4.1 (highly contaminated soils). The results differ when using targeted amplicon sequencing of the 16S rRNA gene – the highest bacterial diversity was found in the highly contaminated soils KCM_1.1 and KCM_1.2, followed by the slightly contaminated soil KCM_3.1. The lowest bacterial diversity was observed in

KCM_3.2 and KCM_2.2. These differences in bacterial diversity are due to the higher taxonomic sensitivity and resolution of targeted amplicon sequencing, while full-length 16S rRNA gene sequencing provides higher taxonomic accuracy. A similar conclusion has been drawn by other authors, who point out that sequencing of the full 16S rRNA gene allows for more precise taxonomic identification, including of rare or weakly represented taxa in bacterial communities, which often remain unidentified when sequencing shorter regions of the 16S rRNA gene (for example, the V4 region) (Wilkinson et al., 2025; Zhang et al., 2023). Therefore, the observed higher bacterial diversity in highly contaminated samples, revealed by targeted amplicon sequencing of the 16S rRNA gene, is not interpreted as a direct biological effect of contamination, but rather as a reflection of the higher resolution of the molecular technique used.

A comparison was made of the abundance/diversity of bacterial communities from the two soil layers. Overall, these indicators had higher values in the surface soil layer, but the difference from the subsurface layer was not statistically significant. This result is consistent with the study of Wilkinson et al. (2025), which shows that bacterial diversity is higher in upper soil layers, although the difference from deeper layers does not always reach statistical significance.

The abundance of fungal communities was determined through the number of copies of the ITS region of the rRNA gene and it was found that this gene was most abundantly represented in the least contaminated soil KCM_3, followed by the moderately (KCM_5.1) and highly (KCM_1.1) contaminated soils (figure 12). With the increasing soil depth, the number of copies decreased, but the difference was not statistically significant. The difference in the number of ITS region copies of the rRNA gene along the gradient of HM contamination was also statistically insignificant. Studies show that the abundance of the ITS region is higher in uncontaminated or slightly contaminated soils and decreases with increasing HM levels (Wang et al., 2025; Li et al., 2025; Bourceret et al., 2016; Petkova et al., 2022) and soil depth (Zhang et al., 2021; Estrada et al., 2024). This leads to an overall change in the structure of fungal communities, since sensitive taxa significantly reduce their relative abundance in favor of resistant ones (Wang et al., 2025).

5.3. Taxonomic composition of soil microbial communities

5.3.1. Taxonomic composition at the phylum level

Within the composition of soil bacterial communities, 12 (from 16S rRNA gene clone libraries) and 15 (from targeted amplicon sequencing of the 16S rRNA gene) bacterial phyla were identified (figures 11A and 13A). The dominant or subdominant phyla, determined by both techniques in terms of relative abundance, were Proteobacteria, Actinobacteriota, Acidobacteriota, Bacteroidota, Chloroflexota, Gemmatimonadota, Planctomycetota and Firmicutes. The presence of these phyla is expected, considering their widespread distribution in soils and adaptability to diverse habitats, including soils contaminated with HMs (Fajardo et al., 2020; Song et al., 2018; Cui et al., 2018; Radeva et al., 2013). The high resistance and adaptability of bacteria from these phyla to the environment are determined by their broad trophic niches, high growth rates and the presence of adaptive mechanisms for HM resistance (Sreedevi et al., 2022; Nnaji et al., 2023). Proteobacteria, Acidobacteriota and Bacteroidota have also been detected in soils with high uranium content (Radeva et al., 2013). Moreover, many representatives of these phyla typically prefer soils with neutral pH (Nacke et al., 2011), as is the case in the area around KCM 2000. According to Zeng et al. (2020), Actinobacteriota, Bacteroidota and Chloroflexota increase their relative abundance in highly HM-contaminated soils, while other phyla such as Proteobacteria and Acidobacteriota decrease their presence. Such a trend was not observed in the soils studied here. Our results show an increase in the relative abundance of Gemmatimonadota and Planctomycetota in KCM_1 soils compared to the others. He et al.

(2020) similarly found an increase in the abundance of Gemmatimonadota and Nitrospirota in soils with higher HM concentrations. The presence of Gemmatimonadetes in highly contaminated with HMs soils has also been confirmed by other studies (Guo et al., 2017; Hemmat-Jou et al., 2018). It is possible that the observed dynamics in the relative abundance of Gemmatimonadota and Planctomycetota with respect to contamination level are determined not only by the metal resistance of these two phyla but also by numerous other environmental factors, both studied and unstudied. For instance, it has been established that the resistance of Proteobacteria and Firmicutes to HMs depends on soil organic carbon and moisture content (Zhao et al., 2019). Some authors (DeBruyn et al., 2011; Fawaz, 2013) associate the prevalence of Gemmatimonadota in extreme soils with their high drought tolerance. On the other hand, the high ecological plasticity of representatives of Planctomycetota is determined by their ability to degrade and utilize highly complex organic compounds that are inaccessible to microorganisms from other taxa (Wiegand et al., 2020).

Regarding the vertical distribution of the phyla, no unified trend was established between the two applied methods. According to the constructed 16S rRNA gene clone libraries, there is a difference in depth distribution for Gemmatimonadota and Actinobacteriota, which show higher relative abundance in the surface soil layer, and for Fibrobacterota, which increase their abundance in the subsurface layer. Gemmatimonadetes and Actinobacteria decrease in relative abundance with increasing soil depth, which is related to the higher content of easily degradable carbohydrate sources in surface layers that these taxa efficiently utilize (Hao et al., 2021). According to targeted amplicon sequencing of the 16S rRNA gene, Acidobacteriota show a more pronounced decrease in abundance with depth. As with the HM contamination gradient, we can assume that specific environmental conditions influence the vertical distribution of taxa and modify community structure. Several studies have reported that the abundance of Acidobacteriota decreases with soil depth across various ecological niches, and that their distribution is closely related to specific environmental conditions such as TOC and pH, which vary with depth (Christiansen et al., 2025; Fierer et al., 2017; Banerjee et al., 2021).

As examples of soils showing an increase in relative abundance of specific phyla, KCM_5, KCM_2 and KCM_1.2 can be mentioned for their higher relative share of Proteobacteria in total bacterial abundance, and KCM_3.1 and KCM_5.2 for their higher relative share of Actinobacteriota. These characteristics of the soils cannot be directly associated with the values of the measured abiotic parameters. The distinguishing features of KCM_5 from the others are its lower pH (6.6-6.8) and organic carbon content, as well as its higher moisture content. It is possible that these factors, along with the moderate HM contamination level, may stimulate the development of Proteobacteria and Actinobacteriota. For the other soils, no significant differences were observed in the values of environmental factors, except for HM content.

The soil fungal communities were entirely dominated by Ascomycota (95.2%-99.79%) (figure 13A). Three additional phyla were identified, among them Basidiomycota and two undefined groups (Fungi_Unclassified and Fungi_phylum_Incertae_sedis), all present at very low abundance levels. Numerous authors have reported the ubiquitous distribution of Ascomycota in soils, considering that their resistance and efficient spore dispersal by wind are the mechanisms behind their wide invasion (Egidi et al., 2019; Tedersoo et al., 2014). In our studies, the relative abundance of Ascomycota did not correlate significantly with the HM levels in the soil. Similar results were obtained by Passarini et al. (2022) when studying HM-contaminated soils in Brazil. They reported that most of the identified OTUs belonged to Ascomycota and only a small fraction (1.1%) to Basidiomycota. The authors found the highest relative abundance of Ascomycota in uncontaminated soil.

5.3.2. Taxonomic composition at class and genus level

Some of the most widely distributed bacterial classes with average relative abundance in the bacterial communities ranging from 5.12% to 24.12% are Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria (Proteobacteria); Actinomycetia, Thermoleophilia and Acidimicrobiia (Actinobacteriota); Clostridia (Firmicutes); Vicinamibacteria (Acidobacteriota); Bacteroidia (Bacteroidota) and Gemmatimonadetes (Gemmatimonadota) (figures 7B and 9B). Some of these classes are dominant according to the analysis of 16S rRNA gene clone libraries (Alphaproteobacteria, Betaproteobacteria, Acidimicrobiia and Clostridia), while others (Alphaproteobacteria, Gammaproteobacteria, Actinomycetia, Thermoleophilia, Vicinamibacteria, Bacteroidia and Gemmatimonadetes) are dominant according to the targeted amplicon sequencing of the 16S rRNA gene. This is expected, since the different methods for analyzing the taxonomic profile of bacterial communities are characterized by varying degrees of taxonomic resolution.

The phyla Proteobacteria and Actinobacteriota contain the highest number of classes with high relative abundance. Within Proteobacteria, the most abundant classes are Alphaproteobacteria and Betaproteobacteria. They are distributed across all soils but prevail in soils KCM_2 and KCM_1.2, which have NPI values ranging from 7.99 to 9.27 (KCM_2) and 23.12 (KCM_1.2). Gammaproteobacteria is also widely and relatively evenly distributed, but the highest abundance for this class was recorded in KCM_5 (NPI: from 2.41 to 2.50). The fact that all classes of the phylum Proteobacteria are distributed along the entire gradient of HM contamination indicates their tolerance to this anthropogenic environmental factor. Interestingly, some classes dominate in soils with an NPI around 10, suggesting that this level of contamination either stimulates these bacterial classes or inhibits others, thereby increasing their proportion in the total abundance. Numerous studies have demonstrated the high resistance of Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria to environmental factors (Tsementzi et al., 2016; Yim et al., 2022), including various pollutants (Song et al., 2018; Cui et al., 2018). The presence of these bacterial classes in soils ensures a number of important ecological processes such as nitrogen fixation, mineralization and denitrification (Alphaproteobacteria), degradation of complex natural compounds (Betaproteobacteria and Gammaproteobacteria) and biodegradation of pollutants (Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria) (Hartman et al., 2022). In previous studies conducted by our research group, it was also found that in soils contaminated with U, Cu, Zn, Pb and As from mining regions in Bulgaria, representatives of the classes Alphaproteobacteria and Betaproteobacteria were dominant (Radeva et al., 2005, 2013). Liao et al. (2007) likewise established the occurrence of these classes in mining areas.

In the present study, Actinobacteriota is represented by three classes – Actinomycetia, Thermoleophilia and Acidimicrobiia, with higher representation of Actinomycetia and Acidimicrobiia in the bacterial communities (each about 9%). Thermoleophilia is represented from 1.8% (16S rRNA gene clone libraries) up to 6.4% (targeted amplicon sequencing of the 16S rRNA gene). Representatives of these classes are resistant to environmental factors due to several of their characteristics, such as high G+C content (>60–70 mol%), rigid cell walls, presence of mycelial growth in some (Actinomycetia), ability to degrade complex organic compounds (Barka et al., 2016), ability to survive in dry, warm and oligotrophic environments (Thermoleophilia), as well as ability to inhabit environments with lower pH values (Acidimicrobiia) (Jones et al., 2025; Gao and Gupta, 2012). Acidimicrobiia is also characterized by a good ability to oxidize iron and sulfur and to transform metal compounds (Hanada et al., 2003). Despite their high resistance, representatives of all these classes occur with higher relative abundance in slightly contaminated soil KCM_3 (Thermoleophilia; 8.62%-14.48%) and in moderately contaminated soil KCM_5 (Actinomycetia and Acidimicrobiia; 10.8%-33.3%). The class Bacteroidia also shows high representation in KCM_5. In contrast, the class Gemmatimonadetes increases its relative abundance in highly

contaminated soil KCM_1.1 up to 24% (16S rRNA gene clone libraries) and in KCM_1 up to 9.1% (targeted amplicon sequencing of the 16S rRNA gene) of the total abundance, compared to the average value for all other soils of 0.54% (clone libraries) and 4.13% (amplicon sequencing), respectively. A similar trend is observed for the classes Chloroflexia and Bacilli, but in the latter case the increased abundance is recorded in KCM_4, where the level of contamination is high (average NPI value: 27.87) but lower than that of soil KCM_1 (average NPI value: 45.28).

The distribution of the classes Myxococcia, Polyangia and Nitrospiria is of particular interest. All of them have low representation (1%-2%) in the individual soils and all are absent from the least contaminated soil KCM_3. We assume that in this case, there is another limiting factor for the development of these classes in KCM_3, apart from the influence of HMs. A similarly low relative distribution (~1.5%) of the class Myxococcota was also established by Jiang et al. (2024) in soils with low levels of HM contamination.

By using targeted amplicon sequencing of the 16S rRNA gene, a number of genera in the soil bacterial communities were identified (figure 10). Many representatives of Alphaproteobacteria (*Sphingomicrobium_483265, Microvirga, Skermanella, Devosia_A_501803, Devosia_A_502124, HRBIN40*) show the highest abundance in slightly (KCM_3) and moderately (KCM_5) contaminated soils, which corresponds to their ecological profile and participation in symbiotic interactions (Liu et al., 2020). For genera from the class Gammaproteobacteria, no strict distribution pattern according to the level of contamination was found, which is consistent with the results of Zhou et al. (2019). Some genera (*SCGC-AG-212-J23, UBA5216*) are ubiquitously distributed; others (*Solirubrobacter, Povalibacter, SCUD01, Nitrosospira, Luteimonas_C_615545*) predominate in slightly or moderately contaminated soils, while others show the highest abundance in highly contaminated soils such as KCM_4 (*Aquicella_A*). This has been confirmed by Zhou et al. (2019), who report that genera from the class Gammaproteobacteria are generalists with high ecological plasticity. Actinobacteriota, in particular the class Actinomycetia, are represented by a number of genera with broad distribution (*Nocardioides_A_392796, Streptomyces_G_399870, Blastococcus*), as well as by those with specific preferences (*Mycobacterium, Corynebacterium*) for soils with low and medium levels of contamination. Within the phylum Actinobacteriota, the class Thermoleophilia is represented by the genus *AC-16*, which develops better in the highly contaminated soil KCM_4. The high resistance of the genera from the phylum Actinobacteriota to HMs is associated with efficient antioxidant systems and detoxification mechanisms (Alvarez et al., 2021). The class Bacteroidia is represented by the genus *Chryseotalea* in KCM_1, and by *Chryseolinea*, *Flavobacterium* and *ELB16-189* in less contaminated soils. In the highly contaminated soil KCM_1, higher abundance of several genera was recorded compared to the other soils. Detected genera include *UBA2421* (class Phycisphaerae, phylum Planctomycetota), *AG11* (class Gemmatimonadetes, phylum Gemmatimonadota), *Sulfotelmatobacter* (class Acidobacteriae, phylum Acidobacteriota) and *Chryseotalea* (class Bacteroidia, phylum Bacteroidota), which suggests the development of mechanisms for resistance to HMs. It is known that *UBA2421* plays an important role in the sulfur and nitrogen cycles and that this activity can be further influenced or even stimulated by the presence of HMs in the soils (Lenferink et al., 2024). In contrast to *AG11*, the genus *JAABRT01*, which also belongs to the class Gemmatimonadetes, develops best in the slightly contaminated soil KCM_5.

The conducted ordination analysis of the taxonomic diversity of bacterial communities (figure 11) at the class level shows a high similarity in the composition of bacterial communities from soils KCM_2 and KCM_4 and a large difference between them and the other bacterial communities, as well as between the communities of soils KCM_3, KCM_5 and KCM_1. We assume that these differences are mainly due to the presence of rarer taxa with low representation. It is likely that soils KCM_2 and KCM_4 are inhabited

by taxa resistant to HMs, but the development of these taxa is already inhibited in KCM_1 by the higher levels of contamination. Considering the results from figure 16, we can assume the existence of a complex effect of environmental factors on the distribution of bacterial genera and their relative abundance within the respective communities.

The conducted PERMANOVA shows that contamination with HMs, and to a much lesser extent soil depth, are significant factors affecting the composition of bacterial communities. In line with these results are the significant correlations between HM content/soil depth and the distribution of certain bacterial classes. These results are also confirmed by the CCA at the genus level (figure 16). A significant positive correlation with the level of contamination is observed in the distribution of the classes Acidobacteriae, Chloroflexia, Gemmatimonadetes, Planctomycetia and the rarer classes Phycisphaerae, Saccharimonadia and Nitrospiria. Negative effects of contamination are observed on Alphaproteobacteria, Bacilli and the rarer classes UBA4738_401450 and Binatia. The specificity and significance of the bacterial community response to the level of contamination are determined by the combined effect of pollutants and environmental factors, which under different conditions may vary. It is important to note that although contamination has been long-term, the selective pressure of HMs on bacterial communities is still evident, representing an external factor in their evolution and adaptation processes to their habitat.

The depth distribution of the representatives of the identified classes is significant only for Acidimicrobiia, Vicinamibacteria and Methylomirabilia. All of them increase their relative abundance with depth in the soil profile. The possible reasons for this type of distribution may vary, for example, preferences for lower oxygen levels, lower contamination levels in the subsurface soil layer, or a combination of these two factors. An increase in the relative abundance of the classes Acidimicrobiia, Vicinamibacteria and Methylomirabilia with soil depth has been established in various studies and is associated with low pH and low levels of oxygen and nutrients (Huber et al., 2022; Li et al., 2022; McReynolds et al., 2024).

A comparative analysis of the results shows that abundance and diversity do not correlate significantly with the level of contamination and the depth distribution of bacteria, whereas the taxonomic composition of the communities shows such a correlation. This seemingly contradictory result suggests that changes in distribution occur only in certain taxa. This gives us reason to assume that changes in the taxonomic composition and structure of bacterial communities are a more sensitive criterion for assessing the impact of HMs compared to indicators of abundance and diversity, even in soils with a decades long history of contamination.

The representatives of the fungal soil communities are grouped into 10 classes, with the most widespread among them being Sordariomycetes, Eurotiomycetes, Dothideomycetes and Ascomycota_cls_Incertae_sedis (figure 13B). Their distribution and structural profile suggest a high degree of resistance to HMs and the ability to develop in extreme habitats at the expense of other taxa (Eurotiomycetes dominate in KCM_1 and KCM_4), as well as a change in abundance depending on the contamination level (Sordariomycetes, Dothideomycetes and Ascomycota_cls_Incertae_sedis). According to the correlation analysis, Eurotiomycetes correlate significantly and positively with the level of contamination, whereas Sordariomycetes and Dothideomycetes are negatively affected (figure 14). Often, the distribution profile and relative abundance of these classes are also associated with the stages of decomposition of dead organic matter in the soil – Dothideomycetes thrive in the initial stages, while Sordariomycetes and Eurotiomycetes dominate in the later stages (Guo et al., 2022). It is possible that this dependence on the state of the dead organic matter in the soil modifies the response to HM contamination. Other authors have also found a wide distribution of Eurotiomycetes in HM-contaminated soils (Ye et al., 2020; Muggia et al., 2017; Passarini et al., 2022). For example, Mohammadian et al. (2017) and Passarini et al. (2022) reported high abundance of the genera *Penicillium* and *Aspergillus*, which belong to the class

Eurotiomycetes and are known to have the ability to deactivate Cr, Ni and other HMs (Iram et al., 2012). In the soils studied by us, *Penicillium* and *Aspergillus* are present, but with higher relative abundance at contamination levels up to NPI: 33.99. With greater soil contamination, their share in total abundance decreases rapidly – from an average of 4.96% for both genera to 1.81% in KCM_1. In the studied soils, the genus with the highest resistance to HMs is *Bacillicladium* (class Eurotiomycetes, phylum Ascomycota), which is present in KCM_1 at 51.68% of the total abundance, in KCM_4 at 30.86%, and in the other soils – on average at 1.98%. In the soils around KCM 2000 – Plovdiv, in addition to the above-mentioned genera, Eurotiomycetes are represented by a large number of genera, most of which (*Knufia, Arachnomyces*, and *Castanedomyces*) predominate in soils with contamination levels up to NPI: 9.27.

The higher sensitivity of Sordariomycetes and Dothideomycetes to HM contamination has also been confirmed by other authors (Sim et al., 2018), but as we have already mentioned, this response may be modified by the condition of the dead organic matter in the soil. The class Sordariomycetes is dominated by the genus *Fusarium*, which is almost ubiquitously distributed and represents on average about 14.98% of the total abundance, but also includes numerous other genera (*Enterocarpus, Neoschizothecium, Myrmecridium, Dothideomycetes, Achroiostachys, Sordariales_gen_Incertae_sedis*, and *Coniochaeta*), which develop mainly in more contaminated soils but up to NPI levels of 33.99. Dothideomycetes is represented mainly by genera with specific distributions, with *Alternaria* being the only genus more widely present in the studied soils, most often associated with the decomposition of dead organic matter in the soil and plant diseases (Pavón et al., 2012). As with the previous classes, there are genera with higher occurrence in less contaminated soils or conversely.

In fungal communities, as in bacterial ones, the level of contamination and, to a lesser extent, the soil depth are significant factors for the distribution of fungal classes. The ordination analysis of the fungal community composition at the class level (figure 14) supports this result. Fungal communities in the most highly contaminated soils (KCM_1 and KCM_4) segregate into a compact group, while the other communities form groups differing in their degree of compactness. Nevertheless, the slightly and moderately contaminated soils are localized within the same quadrant of the ordination plot, which suggests relatively similar taxonomic composition and structure of their communities. These results indicate that the representatives of the above-mentioned fungal classes have the potential to withstand contamination stress, but this potential is limited by the contamination level and is probably modulated by specific environmental factors. The correlation analysis does not show a significant relationship between soil depth and changes in the abundance of fungal classes. This is confirmed by a number of studies showing that the taxonomic profile of fungal communities changes significantly under the influence of HMs, while soil depth has no effect or only a weak and statistically insignificant one (Wang et al., 2021; Zhou et al., 2019; Rajapaksha et al., 2004).

In conclusion, it can be stated that the taxonomic analysis of soil microbial communities reveals differences in their composition and structure along the HM contamination gradient. There are classes and their respective genera that are more widely distributed, suggesting good adaptation to environmental factors as well as to the HM content in the soil. For many of these taxa, however, the distribution is limited to contamination levels up to NPI 9.27, and only for some up to NPI 33.99. Very few taxa (assessed mainly at the genus level) have managed to overcome the toxicity of HMs and to develop even in soil KCM_1.1 with NPI 67.43.

Depending on the pattern of the taxonomic profile and changes in the community structure, we can distinguish sensitive, tolerant and resistant classes in both bacterial and fungal communities. This classification is subjective because it is defined by the level of contamination (low, medium, and high – in three degrees of NPI), but it also depends on the modifying effects of specific environmental factors. The

sensitive taxa include the bacterial classes Thermoleophilia and Bacteroidia, as well as the fungal class Ascomycota_cls_Incertae_sedis; the tolerant bacterial classes include Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinomycetia and Vicinamibacteria, and in the fungal communities – the classes Dothideomycetes and Sordariomycetes; the resistant classes are Gemmatimonadetes, Chloroflexia and Bacilli among the bacterial communities, and Eurotiomycetes among the fungal ones. Bacterial genera with a high degree of resistance were identified in KCM_1, and these are *UBA2421* (Phycisphaerae, Planctomycetota), *AG11* (Gemmatimonadetes, Gemmatimonadota), *Sulfotelmatobacter* (Acidobacteriae, Acidobacteriota) and *Chryseotalea* (Bacteroidia, Bacteroidota). Highly resistant fungal genera include *Bacillicladium* (Eurotiomycetes, Ascomycota), *Curvularia* (Dothideomycetes, Ascomycota) and to a lesser extent *Aspergillus* and *Penicillium* (Eurotiomycetes, Ascomycota), and *Fusarium* (Sordariomycetes, Ascomycota). All of these taxa not only overcome the toxicity of HMs but also manage to increase their abundance and alter the structure of microbial communities in contaminated soils to levels that are determined by their ecological and physiological potential.

5.3.3. Taxonomic composition at the species level

The construction of 16S rRNA gene clone libraries allows for the identification of 16S rDNA sequences at the species level (~1500 bp), based on which a phylogenetic analysis was performed (figure 8). The phylogenetic analysis of the 16S rDNA sequences identified strains such as *M. neuiana*, strain PTW21 [KCM_1_1_49 (OQ422585)], *H. palleronii*, strain NBRC 102513 [KCM_1_2_3 (OQ422632)] and *P. rivuli*, strain FT92W [KCM_4_1_72 (OQ422734)] (Proteobacteria, Betaproteobacteria), *P. daejeonesis*, strain NBRC 101159 [KCM_5_1_108 (OQ422743) and KCM_5_1_114 (OQ422746); Proteobacteria, Gammaproteobacteria], as well as *P. endophyticus*, strain PECAE04 [KCM_3_1_101 (OQ422910)] (Firmicutes, Bacilli). The above-mentioned bacterial species are distributed in soils with different levels of contamination, which confirms their ability to survive in extreme environments due to the presence of genes encoding HM resistance (Holochova et al., 2020; Li et al., 2022). In the slightly contaminated soil KCM_3.1, 16S rDNA sequences homologous to *A. ramosus*, strain DMS 43045 [KCM_3_1_9 (OQ422872)] were identified, indicating a low tolerance of this species to HMs.

The phylogenetic analysis of the 16S rDNA sequences identified key bacterial species with high taxonomic similarity (>99% homology) to strains belonging mainly to the phyla Proteobacteria and Firmicutes. The species identified in highly contaminated soils are potential bioindicators of a high level of anthropogenic HM contamination. At the same time, the identification of the species *A. ramosus* only in slightly contaminated soils indicates its sensitivity to HMs. Therefore, this species can be classified as a sensitive bioindicator, whose absence signals a deterioration in environmental quality.

5.4. Predicted mechanisms of resistance in bacterial communities

The conducted metagenomic analysis allowed us to establish predicted pathways of acquired resistance in the bacterial communities, using the available KEGG Orthology (KO) platform. No significant linear correlation was found between the level of contamination and the number/frequency of occurrence of metabolic pathways associated with resistance. This means that bacterial communities that have undergone long-term selection due to prolonged contamination are resistant to HMs regardless of the specific level of pollution. These include, for example, bacterial communities capable of detoxifying HMs present in the soil (Cu, Zn, Cd, As), as well as those capable of handling Hg. The main mechanisms of bacterial resistance to HMs identified are the synthesis of enzymes contributing to detoxification, as well as proteins with various functions – regulators, expressors, transport systems and others.

The highest frequency of genes related to heavy metal resistance was found in highly contaminated soils from the KCM 2000 area, as a result of long-term pollution. A total of 26 enzymes and proteins with varying abundance were identified in the studied soils, all of which participate in HM removal (figure 17). These enzymes are key components of the mechanisms of resistance to toxic metals. Among them, the three most widespread proteins (K15726, K07240 and K16264) are associated with efflux systems responsible for HM expulsion. Other KEGG pathways found at high frequency in the highly contaminated soils (e.g., K12951, K15726, K15725, K07665, K07787 and K07798) are associated with the detoxification of Cu, Co/Ni and Cr. It has been established that, after modification, these pathways can be used by bacteria for protection against Zn, Cd and Pb (Nies, 2003). Some of these pathways serve as basic cellular defense mechanisms against HMs, while others are highly specialized and occur only in well-adapted bacteria (Nies, 2003). Feng et al. (2018) also found an increase in the frequency of KEGG pathways related to the metabolism of amino acids, fatty acids and nucleotides in soils contaminated with Cd. They suggest that microorganisms in contaminated soils adapt their metabolism to withstand the toxic effects of Cd, which is consistent with our results showing adaptive changes in the predicted function of soil microbial communities. Goswami et al. (2022) identified direct correlations between the concentrations of Pb, Mn and Zn and the abundance of KEGG pathways related to genes for antibiotic resistance and HM resistance, which is in agreement with our results regarding the influence of HMs on bacterial communities and their adaptation mechanisms.

5.5. Functional characteristics of soil microbial communities

To determine the influence of HMs on microbial communities, their functional activity was assessed through the analysis of soil enzyme activity (fungal and bacterial) and through the analysis of the bacterial potential to degrade naturally occurring carbon sources of different chemical composition.

It is known that the main producers of soil enzymes are soil fungi, but also, to a lesser extent, bacteria and vegetation (Veena et al., 2011; Güsewell and Gessner, 2009; Burns et al., 2013). In this study, the activities of key enzymes from various metabolic pathways were determined (Dha, BGl, Ur, AcP, AlP Ars), as well as the total enzymatic activity as a result of their combined action. The total enzymatic activity varies among soils and between soil layers. PERMANOVA showed that the level of contamination and soil depth are factors influencing the enzymatic activities in the studied soils. SIMPER demonstrated an overall dissimilarity of about 30%, mainly maintained by Dha and BGl (combined contribution to total dissimilarity around 65%). Numerous studies have investigated the sensitivity of enzymes as bioindicators of soil health and the toxic effects of HMs and many of them identify Dha as one of the most sensitive enzymes (Yang et al., 2016; Kenarova and Radeva, 2010; Kenarova et al., 2013; Palov et al., 2020).

The soils KCM_2 (enzyme activity index: 9.59±2.75) and the surface soil layer as a whole showed the highest overall activity. The soils KCM_1 showed the lowest enzymatic activity (5.19±0.87). The high activity in KCM_2 is difficult to explain, but we assume that the contributing factors may be: high phosphate content, TOC and moisture, some of the highest fungal abundance (OTUs), high fungal diversity (the Shannon index having the highest value in KCM_2.1), as well as the possibility that lower levels of contamination (NPI: 7.99-9.27) stimulate microbial activity. CCA confirmed these results, additionally indicating that the content of HMs is also a significant factor.

A more detailed analysis of the effects of soil parameters, including HMs, on the carbon cycle was performed using the Biolog Ecoplate test. Again, as with the enzyme study, the highest metabolic potential was found in KCM_2 and the lowest in KCM_1 (figure 19). There was no statistically significant difference in metabolic potential between the two soil depths. The relatively similar activity of bacterial communities

indicates that they have the potential to compensate, to a certain extent, for the effects of intoxication through their metabolic plasticity. Many species utilize the same carbon source, which is achieved through changes in community composition, where sensitive taxa are replaced by resistant ones. Therefore, bacterial activity can be stimulated by specific environmental factors, which can partially mask the effects of toxicity.

The metabolic profiles of utilization of different groups of carbon compounds showed that the most preferred are polymeric (Polym) carbon sources, followed by carbohydrates (CH) and amino acids (AA) (figure 20). This is the natural order of preference, as microorganisms are adapted to the available environmental carbon sources, predominantly polymers, and because the use of these sources generates the greatest amount of energy (Martinez-Toledo et al., 2021; Xia et al., 2022). We assume that contamination requires higher energy yields to maintain metabolic pathways for HM deactivation and bacteria therefore shift toward the degradation of carbohydrates, which have the highest energy value per molecule of degraded substrate. Amino acids, in addition to being carbon sources, are also sources of nitrogen. A recent study (Chinthalapudi et al., 2023) also reported that microbial communities adapted to stressful environments show a preference for amino acids. A similar trend of increased utilization of Polym, CH and AA in soils contaminated with HMs has been observed by other authors as well (Feigl et al., 2017; Pradhan et al., 2019; Candan et al., 2021). Such a defensive mechanism is activated under stress conditions, when bacterial communities redirect their metabolism toward preferential utilization of specific categories of carbon sources (Kenarova et al., 2014; Aleksova et al., 2021). Furthermore, previous studies by our research team (Kenarova et al., 2013) did not find a significant relationship between HMs and metabolic activity. These results emphasize the importance of factors such as pH and TOC content, which can mitigate the toxic effect of HMs.

6. Conclusions

1. The pattern of change in the taxonomic profile of microbial communities along the gradient of heavy metal contamination shows that pollutants significantly affect the composition, structure and function of the soil microbiome and bioindicator taxa have been identified for different levels of contamination.
2. Specific abiotic factors – soil moisture, organic carbon and phosphates – modify the intoxicating effect of heavy metals on microbial communities.
3. The annotated 26 metabolic pathways related to the adaptation of bacterial communities to heavy metals in soils do not correlate significantly with the level of contamination, which indicates a high potential for microbiome resistance in the studied soils.
4. The established high microbial abundance and diversity in all soils confirm the resilience of the microbiome to heavy metals, which serves as a prerequisite for the realization of the main ecological processes in anthropogenically contaminated soils.
5. The identified presence of bioindicator taxa such as Chloroflexia, Gemmatimonadetes, Planctomycetia and Bacillicladium determines the specific taxonomic profile associated with resistance to heavy metals and provides a methodological possibility for rapid indication of the effects of heavy metal contamination on soil microbial communities.
6. The metabolic profiles of heterotrophic bacteria shift toward more active degradation of complex carbohydrates, accumulating more energy required for their resistance.
7. Dehydrogenase and beta-glucosidase have been established as indicators of changes in contaminated soils, showing preserved functional activity in soil processes even at high contamination levels.

7. Contributions of the dissertation

Contributions of original character

1. The first comprehensive study of the microbiome in heavy metal contaminated soils in Bulgaria was conducted, which determined significant changes in its composition, structure and function along the contamination gradient.
2. The first documented presence of bioindicator species – *Neobacillus niacini, Massilia neuiana* and *Bacillus pseudomycoides*, associated with resistance to heavy metals and *Agromyces ramosus* for low contamination in soils from the area of KCM 2000, Plovdiv.
3. Demonstration of key taxa in the soil microbiome in relation to adaptation to heavy metals:
    - ✓ Resistant – representatives of bacterial classes: Gemmatimonadetes, Chloroflexia and Bacilli and the fungal class Eurotiomycetes.
    - ✓ Tolerant – representatives of bacterial classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinomycetia and Vicinamibacteria and fungal classes Dothideomycetes and Sordariomycetes.
    - ✓ Sensitive – representatives of bacterial classes Thermoleophilia and Bacteroidia and the fungal class Ascomycota_cls_Incertae_sedis.

Confirmatory contributions

1. Long-term soil contamination with heavy metals leads to degradation of soil health in agricultural lands and exerts selective pressure that shapes the structure of the microbiome and its functional potential, resulting in the dominance of resistant and adapted microbial taxa.
2. Key taxa characteristic of heavy metal contaminated soils have been confirmed the presence of bacterial phyla Proteobacteria, Acidobacteriota and Actinobacteriota and the fungal phylum Ascomycota.

8. References

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Publications on the dissertation topic

1. **Nikolova, R.**, Kenarova, A., Boteva, S., Dinev, N., Radeva G. 2024. Diversity and structure of soil bacterial communities in the area of non-ferrous metal plant revealed by 16S rRNA gene retrieval. Comptes rendus de l'Académie bulgare des Sciences, vol. 77 (8), 1260–1268, IF=0.3, https://doi.org/10.7546/CRABS.2024.08.18, Q3
2. **Nikolova, R.**, Boteva, S, Kenarova, A., Dinev, N., Radeva, G. 2023. Enzyme activities in soils under heavy metal pollution: a case study from the surroundings of a non-ferrous metal plant in Bulgaria. Biotechnology & Biotechnological Equipment, 37 (1), doi.org/10.1080/131028the 18.2022.2149348, IF=1.762, Q3
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3. **Nikolova, R.**, Petkova, M., Dinev, N., Kenarova, A., Boteva, S., Berov D, Radeva G. 2022. Correlation between bacterial abundance, soil properties and heavy metal contamination in the area of non-ferrous metal processing plant, Southern Bulgaria. BioRisk (17), pp. 19–30.DOI: 10.3897/biorisk.17.77458, SJR=0.17, Q4
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4. **Nikolova, R.**, Radeva, G., Kirilov, K., Dinev, N., Boteva, S., Kenarova, A. 2023. A total of 166 verified 16S ribosomal RNA gene partial sequences have been annotated in GenBank, accession numbers OQ422575–OQ422639; OQ422707–OQ422751; OQ422861–OQ422916.

Other publications

1. Palov, D., **Nikolova, R.**, Aleksova, M., Dinev, N., Boteva, S., Kenarova, A., Dimitrov, R., Radeva, G. 2019. A total of 99 verified 16S ribosomal RNA gene partial sequences have been annotated in GenBank under accession numbers MN708054–MN708152.
2. Palov, D., Aleksova, M., **Nikolova, R.**, Dinev, D., Kenarova, A., Boteva, B., Dimitrov, R., Radeva, G. 2020. Relationships between soil microbial activity, bacterial diversity and abiotic factors along the heavy metal contamination gradient. Ecologia Balkanica, 12(3), 31–39.

Conference participations

1. **Николова, Р.**, Радева, Г. Таксономичен и функционален анализ на почвения микробиом по концентрационния градиент на замърсяване с тежки метали. Първо национално лятно училище по природни продукти с приложение в медицината, 28.06-02.07.2021, Творчески дом на БАН, Варна. Oral presentation.
2. **Nikolova, R.,** Aleksova, M., Dinev, N., Kenarova, A., Boteva, S., Berov, D., Radeva, G. Correlation between bacterial abundance, soil properties and heavy metal contamination in the area of non-ferrous metal processing plant, Southern Bulgaria. International Seminar of Ecology – 2021 "Current trends of ecology", 29-30 September 2021, Online seminar. Oral presentation.
   Award Certificate for the best oral presentation
3. Radeva, G., **Nikolova, R.**, Gatev, E., Kenarova, A., Petkova, M., Dinev, N., Boteva, S. Impact of heavy metal pollution on soil bacterial community structure in the area of a non-ferrous metal processing plant, Southern Bulgaria.". International Conference on DNA Barcoding and Biodiversity (ICDBB). 25-27 May 2022. Sofia, Bulgaria. Poster.
4. **Nikolova, R.**, Kenarova, A, Boteva, S, Dinev, N, Gatev, E., Radeva, G. Composition and functioning of the bacterial communities in heavy metal contaminated soils: A case study from the surroundings of a non-ferrous metal plant in Bulgaria". 100 anniversary of the birth of Acad. Roumen Tsanev conference, 5-7 October 2022, Sofia. Oral presentation.
5. **Николова, Р.**, Кирилов, К., Динев, Н., Кенарова, А., Радева, Г. Два молекулярни подхода за изучаване структурата на бактериалните съобщества. Трето национално лятно училище по природни продукти с приложение в медицината, 27.06-02.07.2023, Бургас. Oral presentation.
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9. Bidzhova, L., Georgiev, S., Radeva, G., **Nikolova, R**. Mercury in soils near a base metal smelting plant, Plovdiv, Bulgaria. 37th International Geological Congress 2024, 25-31 August 2024, Busan, South Korea. Poster.