BioRisk 17: 19–30 (2022)
doi: 10.3897/biorisk.17.77458
https://biorisk.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
BioRisk

Correlation between bacterial abundance, soil properties and heavy metal contamination in the area of non-ferrous metal processing plant, Southern Bulgaria

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Academic editor: Michaela Beltcheva | Received 1 November 2021 | Accepted 20 December 2021 | Published 21 April 2022

Citation: 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. In: Chankova S, Peneva V, Metcheva R, Beltcheva M, Vassilev K, Radeva G, Danova K (Eds) Current trends of ecology. BioRisk 17: 19–30. https://doi.org/10.3897/biorisk.17.77458

Abstract

In the present study, the correlation between bacterial abundance and soil physicochemical properties along the heavy metal contamination gradient in the area of non-ferrous metal processing plant was assessed. Our results showed that bacterial abundance (number of heterotrophic bacteria and number of 16S rRNA gene copies) decreased with 45–56% (CFU) and 54–87% (16S rRNA gene) along the Zn, Pb and Cd contamination gradient. The total bacterial abundance (16S rRNA gene) increased exponentially in contrast to the abundance of heterotrophic bacteria. The reduction of bacterial abundance in heavily contaminated soil indicated that the soil properties (soil pH, total organic carbon, inorganic ions, soil texture) could modify the effects of heavy metals and the response of microorganisms to that stress in long-term contaminated soils.

Keywords

Bacterial abundance, 16S rRNA gene, heavy metals, soil contamination, soil properties

Introduction

Soil sustains a great abundance and diversity of microorganisms, which modify its physical and chemical environment and play an essential role in the mineralization of organic matter and nutrient recycling (Martinez-Toledo et al. 2021). Microbial communities are strongly susceptible to soil physicochemical properties and to the effect of various soil pollutants, such as heavy metals (HMs). HMs are one of the most common pollutants, which accumulate in soils of industrial and mining areas. The most common HMs found in contaminated sites are Zn, Cd and particularly Pb (Wuana and Okieimen 2011; Fajardo et al. 2019). Long-term contamination with HMs is a threat to human health and ecosystems due to their non-biodegradability, bioaccumulation, environmental stability, persistence and biotoxicity characteristics (Ali et al. 2021).

Previous studies showed that HMs severely affect soil microbial communities by reducing their diversity (Chodak et al. 2013; Zampieri et al. 2016), richness (Cui et al. 2018), microbial biomass, metabolic activity (Chen et al. 2014; Hong et al. 2015; Zampieri et al. 2016; Fajardo et al. 2019) and by altering their structure (Cui et al. 2018; Feng et al. 2018; Zhang et al. 2018). Recently, many studies were focused on the ecological effects of HMs on microbial community structure and diversity using next-generation DNA sequencing technologies (NGS) (Gołębiewski et al. 2014; Fajardo et al. 2019; Jiang et al. 2019; Xiao et al. 2019; Zhao et al. 2019; Huang et al. 2021). Gołębiewski et al. (2014) found that the diversity and abundance of soil microorganisms near a Pb-Zn mining area have been reduced and that Zn was the largest selective factor. Zhao et al. (2019) reported that HMs (Cu, Zn, Pb) affected the abundance and structural diversity of microbial communities in the mining area. Fajardo et al. (2019) showed significant phylogenetic and functional shifts in the bacterial community during the soil exposure to Pb, Cd, and Zn. Xiao et al. (2019) revealed that the bacterial community structure was mainly altered by soil organic matter, HMs (Cr) and pH. According to Huang et al. (2021), pH and HMs (Cr, Cu, Ni, and Zn) were among the most powerful factors, which change the community structure in HM contaminated soil under remediation.

Many studies reported that soil physicochemical properties (soil pH, soil texture, organic matter, etc.) moderate HMs' toxicity and therefore, HMs play a key role in shaping the community diversity and structure (Wang et al. 2022).

Taking into consideration that soil is highly heterogeneous, it is necessary to investigate the microbial communities at different sites and scales (Wang et al. 2022). In this term, the aim of this study was to assess the correlation between soil bacterial abundance and soil physicochemical properties, including HM content along the Zn, Pb and Cd contamination gradient in the area of non-ferrous processing plant KCM-2000. KCM-2000 is the largest lead-zinc smelter in the country, located in the vicinity of Plovdiv city, Southern Bulgaria. We hypothesized that long-term HM contamination reduced the abundance and changed the composition of indigenous soil bacterial communities, and these effects might be modulated by the local soil properties.

Materials and methods

Study area and soil sampling.

The study area is located in the region of a non-ferrous metal plant KCM 2000- Plovdiv, Southern Bulgaria (42°03'40.8"N, 24°48'52.0"E) (Fig. 1). Topsoil samples (0–20 cm) were collected in June 2020 along a gradient of contamination with Zn, Pb and Cd. Five points have been selected from a monitoring map, considering the direction of spread of the diffuse pollution as follows: KCM_1, named "Green belt of decorative trees", (42°03'31.68"N, 24°49'19.2"E), located at a distance of 0.5 km – south of the smelter; KCM_2 (42°03'5.76"N, 24°49'19.6"E) – 2 km south of the smelter; KCM_3 (42°02'6"N, 24°49'19.2"E) located close to the village of Dolni Voden – 3 km south of the smelter; KCM_4 (42°03'31.68"N, 24°49'45.12"E) – 1 km south-east of the smelter; and KCM_5 (42°04'23.52"N, 24°49'45.12"E) – 1 km east of the smelter. Five subsamples per site were pooled and used for further analyses. At all sites, the soil was classified as alluvial, being crop managed, except at KCM_1, where it was classified as technogenic.

Soil physicochemical properties and heavy metal content.

Soil pH was measured in 0.1M $CaCl_2$ according to ISO 10390:2005. Soil texture was determined by the Kachinsky method (1958). The total organic carbon (TOC) was determined according to Chen et al. (2014). Soil nitrate (NO_3-N) and ammonium (NH_4-N) nitrogen and inorganic phosphates (P_2O_5) were deter-

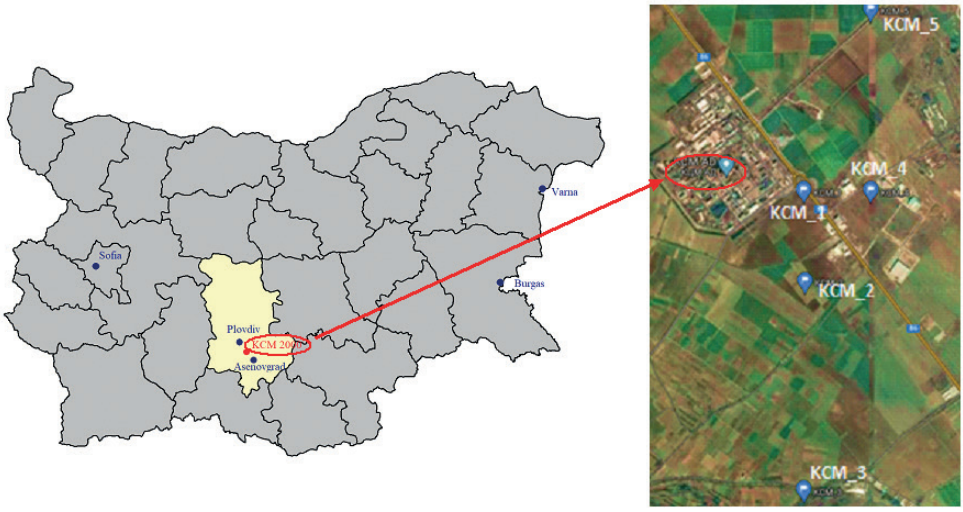

Figure 1. Map of the study area in Southern Bulgaria and sampling sites.

mined according to the methods of Keeney and Nelson (1982), and Olsen (1982), respectively. Soil moisture (SM) was calculated after oven drying (105 °C). The concentration of heavy metals was measured by ELAN 5000 Inductively Coupled Plasma Mass Spectrometer (Perkin-Elmer, Shelton, CT, USA) according to ISO 11047:1998 after soil decomposition by *aqua regia* (total HMs) and soil extraction with 0.01M $CaCl_2$ (bioavailable forms of HMs). Nemerow's pollution index (NPI) was calculated to evaluate the overall pollution of heavy metals in the soil samples (Zhao et al. 2019).

Enumeration of heterotrophic bacteria.

The bacterial abundance was estimated by the use of colony-forming units (CFUs) in serial dilution in R2A medium at 25 °C for 2 days. The selected dilution from each test sample was 10^{-4} and it was plated in triplicate. For this analysis, we used 10–100 colonies per plate.

DNA extraction.

The genomic DNA was extracted from 0.5 g soil using the E.Z.N.A DNA soil kit (Omega Bio-tek, USA) using the manufacturer's recommended protocol. The soil DNA quality was controlled by a spectrophotometer (NanoDrop 1000, ThermoScientific, USA) and agarose gel electrophoresis.

Quantitative PCR (qPCR) of the 16S rRNA gene

Bacterial abundance was quantified by real-time quantitative PCR (qPCR) with bacterial universal primer pairs Eub338f (5'-ATTACCGCGGCTGCTGG-3')/Eub518r (5'-ATTACCGCGGCTGCTGG-3') for 16S rRNA gene (Fierer et al. 2005). The qPCR reactions were set up using iTaqTM Universal SYBRGreen Supermix (BioRad) as described in Aleksova et al. (2020) and PCR efficiency was 90% (R^2 =0.9879).

Statistical analyses

Soil properties and HM concentrations were compared using principal component analysis (PCA). Prior to the analysis the data was normalized and checked for outliers. Linear correlations between the resulting indicator of pollution and the copies of 16S rRNA gene found in each sample were plotted and evaluated (r2 metric of plotted trendline). Additionally, the significance of the correlation between the two variables was evaluated with the Student T-test.

The PCA statistical analyses were carried out using Primer 7.0. Univariable statistical correlations were tested using STATGRAPHICS Centrion XVII software package. (Karamfilov et al. 2019).

Results

Environmental variables

The values of studied soil properties are shown in Table 1. The soils were determined as sandy loam textured. The soil pH was neutral (pH 6.7 to 7.2) and the total organic matter content (TOC) ranged from 6.45 g kg^{-1} to 14.07 g kg^{-1}. Soils were abundant with inorganic nitrate (especially KCM_1 and KCM_2) and inorganic phosphates. Probably, the high NO_3-N concentration in KCM_1 and KCM_2 was due to soil fertilization.

The HM concentrations at KCM_3 were under (Zn) and slightly higher (Pb and Cd) than the maximum permissible concentration (MPC) allowed under Bulgarian Regulation 3/2008 (http://eea.governement.bg/bg/legislation/soil), and this sample was considered as a control in our study. Pb was the most serious soil pollutant, and its concentration was over 100 (KCM_1), 57 (KCM_4), 13.7 (KCM_2) and 3 (KCM_5) times higher than the guideline limit. Cd was the other most serious soil pollutant and its concentrations exceeded the MPC in the following order: KCM_1 (92.45 times) >KCM_4 (43 times) >KCM_2 (7.85 times) >KCM_5 (4.0 times). The order of Zn soil contamination comparing to MPC was: KCM_1 (29.5 times) >KCM_4 (21.0 times) >KCM_2 (4.8 times) >KCM_5 (2.0 times). Nemerow's Pollution index (NPI) assessed the overall level of soil contamination (Zhao et al. 2019), and the soils were classified as heavily contaminated (NPI>3), except KCM_3, which had precautionary values of contamination (Table 1).

Table 1. Soil physicochemical properties and concentrations of heavy metals (total and bioavailable forms) in the area of KMC-2000.

Soil parameter	Soils				
	KCM_1	KCM_2	KCM_3	KCM_4	KCM_5
pH	7	7.1	7.2	6.7	6.8
Sand (%)	17.6	53.6	47.1	49.0	51.7
Silt (%)	39.2	30.9	31	30.8	36.2
Clay (%)	43.3	15.5	21.9	20.2	12.1
Soil moisture (SM) (%)	16.7	12.3	9.3	14.7	22.7
TOC (g kg^{-1})	9.65	14.07	6.45	12.33	7.035
NO_3-N (mg g^{-1})	43.38	16.32	3.01	5.13	†ND
NH_4-N (mg g^{-1})	6.62	5.13	3.26	2.25	2.22
P_2O_5 (mg kg^{-1})	5.69	24.02	7.42	6.89	†ND
Zn (mg kg^{-1})	9452	1558.2	216.2	6872	740
Pb (mg kg^{-1})	11569	1370.1	135.6	5723	335
Cd (mg kg^{-1})	184.9	15.7	3.9	86.2	9.3
$Zn_{b‡}$ (mg kg^{-1})	8.2	0.1	0.1	3.3	0.3
$Pb_{b‡}$ (mg kg^{-1})	2.6	0.2	0.9	0.2	0.8
$Cd_{b‡}$ (mg kg^{-1})	9	0.2	0.5	1.1	0.4
NPI§	73.27	8.74	1.00	37.46	3.11

†ND – No data; ‡$_b$ – Bioavailable forms of the heavy metals; §NPI – Nemerow Pollution Index.

Soil bacterial abundance

The results from the bacterial abundance of heavily contaminated soils were compared as a percent to KCM_3, which was a control soil in the experiment (Fig. 2). The highest number of cultivable heterotrophic bacteria (CFU) and 16S rRNA gene copies were reported for KCM_2. This was the only site, where bacterial abundance was around 27% (CFU) and 55% (16S rRNA) higher than that of the control (KCM_3). The lowest bacterial abundance was detected in the most contaminated site KCM_1, where it decreased by 56% (CFU) and 87% (16S rRNA gene copies) compared to the control soil. The soil bacterial abundance in KCM_4 and KCM_5 decreased by 47% (CFU) and 64% (16S rRNA) for KCM_4, and by 45% (CFU) and 17% (16S rRNA) for KCM_5 compared to KCM_3.

Correlation between soil properties and heavy metals.

Soil properties (total organic content, inorganic ions, soil particles of silt, clay and sand) and HM concentrations in soils were compared using principal components analysis (PCA) and the results are presented in Fig. 3. PC1 explained 65.7% of the total soil variation and showed a significant negative correlation with HM concentrations (total and bioavailable forms), concentrations of nitrate, nitrogen, soil pH and silt particles (Table 2). PC2 explained 17.7% of the total soil variation and positively correlated with sand particles and negatively with TOC and phosphates. PCA ordination showed a clear gradient of contamination level and differences in soil char-

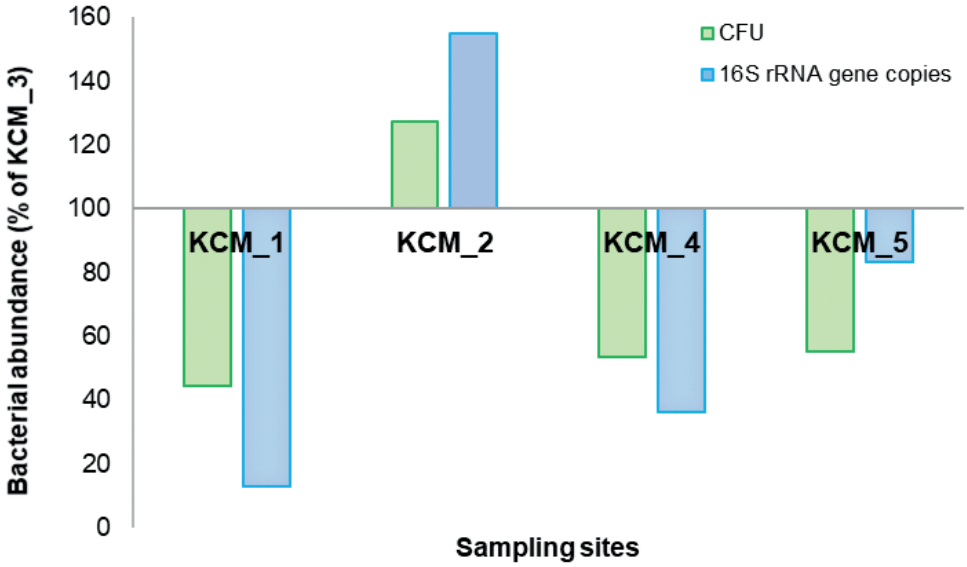

Figure 2. Soil bacterial abundance (% of KCM_3) of cultivable heterotrophic bacteria (CFU) and 16S rRNA gene copies.

Table 2. PCA axes scores of measured soil variables.

Variable	PC1	PC2	PC3	PC4
Variation explained (%)	65.7	17.7	13.4	3.2
pH	-0.001	**-0.308**	**-0.618**	**-0.327**
TOC	-0.011	**-0.501**	**0.437**	0.205
Sand	**0.324**	-0.047	0.119	0.110
Silt	-0.248	**0.266**	-0.137	**0.708**
Clay	**-0.316**	-0.027	-0.102	**-0.358**
Zn	**-0.284**	-0.019	**0.357**	-0.211
Pb	**-0.313**	-0.028	0.219	0.127
Cd	**-0.316**	0.000	0.197	-0.117
Zn_b	**-0.321**	0.032	0.156	-0.119
Pb_b	**-0.296**	0.142	-0.276	0.067
Cd_b	**-0.328**	0.019	-0.057	0.037
NO_3-N	**-0.305**	-0.216	-0.080	0.199
NH_4-N	-0.244	**-0.357**	-0.240	0.251
P_2O_5	0.068	**-0.620**	-0.022	0.116

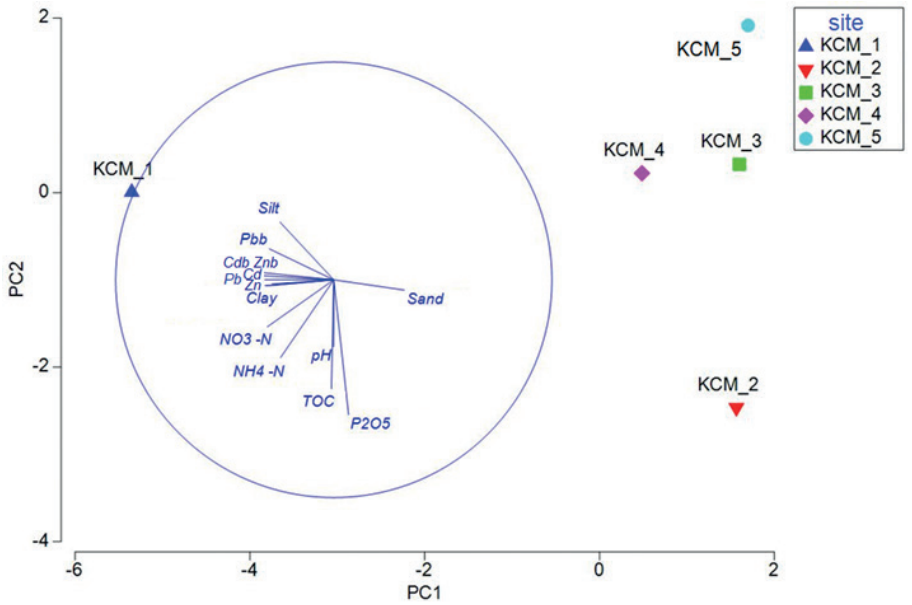

Figure 3. Principal component analysis of soil properties and heavy metals in the area of KCM-2000. Principal components axis 1 (PC1) explains 65.7% of the total soil variation and PC2 – 17.5%.

acteristics between different sampling sites (Fig. 3, Tables 2, 3). Thus, the resulting PC1 score for each sampling site can be regarded as an integral index of heavy metals pollution throughout the study area. Based on this evaluation, the soils of KCM_3 (PC1=1.6) and KCM_2 (PC1=1.57) were qualified as the least impacted by HMs, followed by KCM_5 (PC1=1.7), and the highly impacted soils of KCM_4 (PC1=0.486) and KCM_1 (PC1=-5.35) (Table 3).

Table 3. PC1 score and 16s rRNA gene copies.

Sample	PC1 score	16S rRNA gene copies $\times 10^{10}$
KCM_1	-5.35	1.49
KCM_2	1.57	17.80
KCM_3	1.6	11.50
KCM_4	0.486	4.17
KCM_5	1.7	9.57

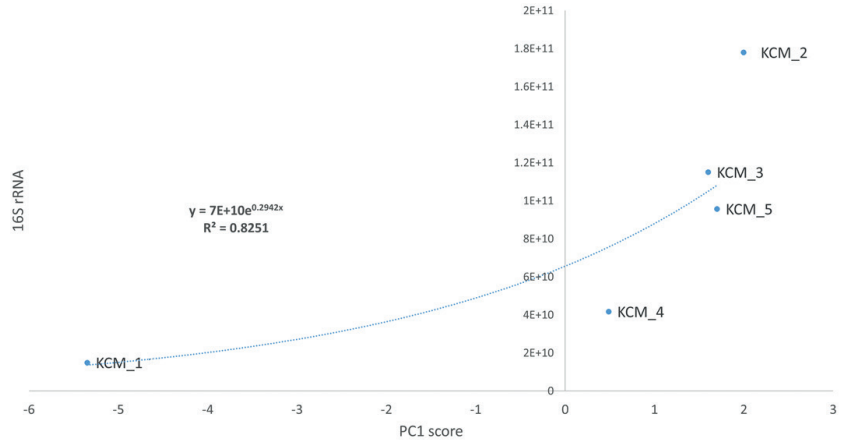

Figure 4. Exponential correlation between integrated HM contamination status (PC1 score) and bacterial abundance (16S rRNA gene copies) in the studied soils (R^2=0.8251) from the area of KCM – 2000.

Correlation between bacterial abundance and soil properties.

The soils had a high abundance of bacteria, whose number varied from 17.80×10^{10} (KCM_2) to 1.49×10^{10} (KCM_1) 16S rRNA gene copies (Table 3). To evaluate the impact of HM contamination on soil bacterial abundance, estimated as 16S rRNA gene copies, an exponential correlation was performed. The good exponential correlation between the values of PC1 pollution score, obtained by the PCA (Table 3), and 16S rRNA gene copies (R^2=0.8251) was demonstrated in Fig. 4. The results indicated both a dramatic decrease of soil bacterial abundance at KCM_1 and its exponential increase in soils along the gradient of contamination with Zn, Pb and Cd. The same analysis was conducted with the abundance of soil heterotrophic bacteria. The influence of increasing heavy metal contamination on microbial abundance was also confirmed by the significant correlation between the 16S rRNA gene copies and the PC1 pollution score (Student T-test, p=0.017).

Discussion

The present study focused on the correlation between bacterial abundance and soil properties in long-term contaminated soils in the area of non-ferrous metal processing

plant KCM-2000. The gradient of Zn, Pb, and Cd concentrations in the soil from KCM_3 to KCM_1 provided a good soil pattern for estimating the changes that occurred in soil bacterial abundance under the power of long-term HM contamination. The soil of KCM_3 was determined in this study as a control (NPI=1.00). In KCM_3, the concentration of Pb was slightly higher than the MPC, and Pb bioavailable forms were equal to that of KCM_5, exceeded by 2.5 times that of KCM_2 and KCM_4, and was by 3.0 times lower than Pb_b of KCM_1.

The distribution of bacterial abundance of unculturable and cultivable bacteria along a gradient of contamination was estimated through quantitative PCR of 16S rRNA gene and numbers of colony-forming units (CFU). In general, the soils of the site of interest showed a high bacterial abundance – 1.49×10^{10}-17.80×10^{10} 16S rRNA gene copies (total bacterial abundance) and 1.30×10^{6} -3.70×10^{6} CFU (abundance of heterotrophic bacteria).

Although, the HM gradient of soil contamination determined a gradient of soil bacterial distribution, only in the case of KCM_2 bacterial abundance was higher (around 55% for 16S rRNA and 27% for CFU) compared to that of the control. We suggested that this inconsistency with the model of general bacterial distribution could be due to the toxicity of the much higher Pb_b concentrations in KCM_3, or attributed to the modulating effects of higher concentrations of TOC, NO_3-N and P_2O_5 in KCM_2 compared to KCM_3 soil. Bacterial reduction in HM contaminated soils varied between 45–56% (CFU) and 54–87% (16S rRNA), except for the relatively low decrease in 16S rRNA gene copies in KCM_5 (17% compared to KCM_3). This fact could be explained by the relatively low level of soil contamination compared to the other studied sites (NPI=3.11). The obtained results were consistent with our previous study, where bacterial abundance (CFU and 16S rRNA gene copies) decreased in long-term contaminated with Cu, Zn and Pb soils in the area of copper mine and smelter (Aleksova et al. 2020; Palov et al. 2020). Similar findings for the decrease of CFU (Pacwa-Płociniczak et al. 2018) and 16S rRNA gene copies (Yin et al. 2015) under a long-term HM pollution in soils were observed by other authors. Fajardo et al. (2019) explained the decrease in bacterial abundance under HMs by a decrease in the metabolic activity of *Bacteria* in microbial soil communities. Other authors showed the opposite trend, which manifested that HMs (Cu, Zn, Pb and Cd) affected slightly the abundance, but strongly the diversity of bacterial communities (Tipayno et al. 2018; Huang et al. 2021).

To elucidate the effects of HMs and soil properties on the distribution of bacteria, an exponential correlation between the values of PC1 scores, and both 16S rRNA gene copies and CFU was conducted. There was found to be a good correlation between soil variables and 16S rRNA gene copies, but a low relationship between soil characteristics and CFU. We assumed that the lack of significant correlation between soil variables and heterotrophic bacteria (CFU) might have resulted from the limitation of cultivation technique or the high ecological tolerance of cultivable representatives of bacterial communities. Some authors (De Leij et al. 1994) suggested that cultivable bacteria could be classified as r-strategic opportunists, which grow fast, tolerate environmental fluctuations and are highly resistant to outside impacts.

Conclusions

The total bacterial abundance (estimated by the quantified 16S rRNA genes) increased exponentially in contrast to the abundance of heterotrophic bacteria, which could be explained by the limitations of the cultivable method. Regarding the statistical analysis, the bacterial abundance, expressed by the 16S rRNA gene copies, was considered as a more valuable indicator of the HM contamination effects on the soil inhabitants in comparison to the heterotrophic bacterial abundance. The reduction of bacterial abundance in heavily contaminated soil indicated that the soil properties (soil pH, total organic carbon, inorganic ions, soil texture) could modify the effects of heavy metals and the response of microorganisms to that stress in long-term contaminated soils. Further studies are needed for investigating the shifts in bacterial community structure in this area in response to the HM contamination gradient.

Acknowledgements

This study was financially supported by the National Research Fund of the Bulgarian Ministry of Education and Science (Grant-DN11/4-Dec.2017).

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