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Relationships between Soil Microbial Activity, Bacterial Diversity and Abiotic Factors Along the Heavy Metal Contamination Gradient

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Abstract. In this study, the relationships between soil abiotic factors, heavy metals content and soil microbial activity, bacterial abundance, bacterial genotype richness and diversity were analysed in three sites along a Cu gradient (from 53 to 860 mg kg -1) and co-contaminants Zn and Pb, located in the region of Zlatitsa-Pirdop valley, Western Bulgaria. Long-term heavy metal contamination had a significant negative effect on soil microbial activity and our results showed that the dehydrogenase activity (DHA) decreased along the contamination gradient with up to 79% compared to the uncontaminated sample. The principal component analysis (PCA) showed that DHA correlated significantly and positively with total bacterial abundance (16S rRNA gene copies) and nitrate ions (NO_3-N), and negatively with soil pH, heavy metals and their bioavailable forms. Bacterial genotype diversity was mainly influenced by abiotic factors such as soil organic matter and sand fraction of the studied sites.

Key words: heavy metals, soil contamination, dehydrogenase activity, bacterial abundance and diversity, 16S rRNA gene.

Introduction

Soil contamination by heavy metals has been of a great concern on a global scale, because of their threats to the environment, food safety and human health. Heavy metals (HMs) are the main contaminants of Bulgarian soils, being widely distributed as a result of agricultural and industrial activities. Many studies underlined that heavy metals and soil abiotic factors altered the microbial communities in different manner concerning their activity, abundance, diversity, and structure (Wang et al., 2007; Zhang et al., 2013; Park et al., 2018, Wiatrowska et al., 2015; Zhao et al., 2019). In general, long-term contamination with heavy metals causes a

reduction in the diversity, richness and activity of soil microbiome (Wang et al., 2007; Park et al., 2018). Since the microbial communities are more sensitive than soil physicochemical properties to human impact, changes in their microbial activity are often considered to be a good and adequate measuring of their response to environmental stress. One of the most commonly used sensitive indicators of microbial functioning in soil is the activity of dehydrogenase complex. Many authors reported a relationship between reduction of dehydrogenase activity and high concentrations of pollutants in soil, which in turn correlates with a decrease in microbial community diversity and biomass (Baćmaga et al., 2015; Wang et al., 2018; Wolińska & Stępniewska, 2012). By searching links between microbial communities and soil functions, many reports assessed the influence of different edaphic factors on the composition and functional diversity of soil microbiome (Chau et al., 2011; Furtak et al., 2019). Recent research works are focused on the combined effects of multiple factors on multiple processes in soil environment - microbial community-function coupling (Zheng et al., 2019).

This study aimed to analyse the relationships between soil abiotic ((pH, TOC, texture, NO3-N, NH4-N, SM, HMs (total and bioavailable forms)) and biotic (DHA, 16S rRNA gene copies, bacterial genotype richness and diversity) properties along a heavy metal contamination gradient. The object of our study was the long-term contaminated soils with Cu, Zn and Pb in the region of Zlatitsa-Pirdop Valley, Western Bulgaria. The Zlatitsa-Pirdop Valley is situated in one of the largest and the richest in copper-gold-pyrite deposit area of Europe, currently being developed by the company of "Chelopech Mining" EAD.

Material and Methods

Study area and soil sampling. The studied area is located in the region of Zlatitsa-Pirdop Valley, Western Bulgaria (Fig. 1). Topsoil samples (0-20 cm) were collected in May 2018 along a gradient of Cu contamination (from 53 mg kg^{-1} to 860 mg kg^{-1}) and co-contaminants Zn and Pb in the vicinity of Chelopech (42.6995°N, 24.0847°E) (Chel_1, Chel_4), Chavdar (42.6599° N, 24.0561° E) (Chav_3) and Karlievo (42.6852° N, 24.1059° E) (Karl_5) villages. Chel_1 was chosen as a control (uncontaminated) site, whereas Chel_4 and Karl_5 were high (Cu - 210 mg kg^{-1}) and very high (Cu - 860 mg kg^{-1}) contaminated sites, respectively (Table 1). Five subsamples per site were pooled randomly and used for further analyses.

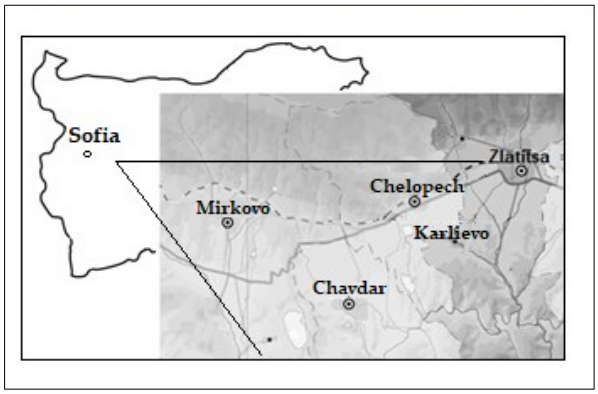

Fig. 1. Region of sampling sites' location (Chavdar, Chelopech and Karlievo villages) in the Zlatitsa – Pirdop valley, Western Bulgaria.

Soil physicochemical properties and heavy metal content. Soil pH was measured in 0.1 M $CaCl_2$ according to ISO 10390:2005(E). 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 were determined according to the methods of Keeney & Nelson (1982) and Olsen (1982), respectively. The soil moisture (SM) was calculated by oven dry method (105 °C). The total content of HMs was estimated after decomposition by *aqua regia*, whereas that of their bioavailable forms was determined after soil extraction with 0.01 M $CaCl_2$. The average pollution index (API) was

calculated to evaluate heavy metal contamination at each site (Hakanson, 1980).

Dehydrogenase activity. We chose dehydrogenase activity (DHA) as a subject of our investigation, because the enzyme is closely related to the soil metabolic activity (Wolińska & Stępniewska, 2012). DHA was assayed by the method of Friedel et al. (1994) based on the reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to INT-formazan (INT-F). The enzyme activity was calculated in micrograms per gram dry soil per hour.

Bacterial abundance. Bacterial abundance (16S rRNA gene copies) was quantified by real-time quantitative PCR (qPCR) with bacterial domain-specific primer pairs Eub338f (5′-ACTCCTACGGGAGGCAGCAG-3′)/Eub518r (5′-ATTACCGCGGCTGCTGG-3′). Q-PCR reactions were set up using iTaqTM Universal SYBR®Green Supermix (BioRad) and the real time qPCR conditions were described in Aleksova et al. (2020).

Bacterial genotype richness and diversity. The values of bacterial richness (Chao1) and diversity (Shannon index and Simpson index of dominance) were calculated (Magguran, 2004) based on the restriction fragment length polymorphism (RFLP) data. Metagenomic DNA was extracted using E.Z.N.A soil DNA kit (Omega Bio-tek, USA) according to the manufacturer's instructions. The 16S rDNA clone libraries were constructed for each sample as described in Radeva et al. (2013) to estimate the bacterial genotype diversity. The obtained clones were subjected by restriction fragment length polymorphism (RFLP) analysis. Briefly, the clones were digested with the restriction enzyme *MspI* (FastDigest MspI, Thermo Fisher Scientific) following the manufacturer's instructions, subsequently were separated on a 3% agarose gel electrophoresis and the obtained patterns were grouped. The dominant 16S rDNA sequences were Sanger sequenced (Macrogen, Europe B.V., Amsterdam, the Netherlands).

Data analysis. One-way ANOVA followed by Tukey's test were performed to examine the differences in the values of soil properties (pH, TOC, SM, NO_3-N, NH_4-N, HPO_4), heavy metals, and microbial variables (DHA, 16S rRNA gene copies, Chao1, Shannon index and Simpson index of dominance). Cluster analysis (Algorithm: UPGMA, Similarity index: Bray-Curtis) and Principal component analysis (PCA) were applied to visualize the relationships between soil abiotic and biotic metrics. Statistical analyses were performed using SPSS for Windows, version 18.0.

Results and Discussion

Soil physicochemical properties, total content and availability of heavy metals

The values of studied soil variables are shown in Table 1. The soils from all sites were determined as loamy sand textured. Soils were acidic, well abundant of organic carbon, inorganic nitrogen (especially Chel_1 and Chav_3) and inorganic phosphates. The high NO_3-N content in soils of Chel_1 and Chav_3 was associated with their land-use manner for annual crop production, where the soil fertilization is a common practice. The concentration of Cu and Pb were under and close to the maximum permissible concentrations (MPC) according to Bulgarian Regulation 3/2008, whereas in Chel_4 and Karl_5, their concentrations exceeded the guideline limit (Table 1). The concentrations of Zn did not exceed the background values in all samples (Table 1). According to the API, Chel_1 (1.3) showed close to the background heavy metal pollution, and was considered as a control in our study. Chav_3 (1.41) showed medium heavy metal pollution, while Chel-4 (3.82) and Karl_5. (11.36) showed high and very high heavy metal pollution (Hakanson, 1980). Bioavailable forms of each HM were below 1.0% of the respective total concentration, except Zn in Karl_5, where its bioavailable forms were calculated to be 1.83% of the total Zn concentration.

Table 1. Soil physicochemical and microbial parameters. *Legend:* According to Regulation 3/2008, the maximum permissible concentrations of HMs (mg kg^{-1}) in arable soils at < 6.00 pH are: Cu ≤ 80, Zn ≤ 200, and Pb ≤ 60. Cu_b, Zn_b, Pb_b – Bioavailable form of the heavy metal; ND – No data; Different letters per row indicate significant differences between the mean values of the respective soil variable ($p<0.05$).

Parameters	Sampling sites			
	Chel_1	**Chav_3**	**Chel_4**	**Karl_5**
Physicochemical				
pH	4.60^{a}	5.30^{b}	4.80^{a}	5.20^{b}
Sand (%)	45.60^{a}	45.00^{a}	50.90^{b}	31.20^{c}
Clay (%)	17.00^{a}	19.20^{a}	18.30^{a}	18.90^{a}
Silt (%)	37.50^{a}	35.80^{a}	30.90^{b}	49.90^{c}
Soil moisture (SM; %)	5.00^{a}	8.60^{b}	4.30^{a}	7.30^{ab}
Total organic carbon (TOC; g kg^{-1})	13.95^{a}	17.44^{a}	24.42^{b}	16.86^{a}
NO_3-N (mg g^{-1})	38.67^{a}	62.41^{b}	2.36^{ce}	8.32^{de}
NH_4-N (mg g^{-1})	1.92^{a}	4.77^{b}	6.13^{bc}	2.60^{ab}
HPO_4 (mg g^{-1})	15.47^{a}	8.91^{b}	13.7^{2ab}	10.90^{ab}
Cu (mg kg^{-1})	53.00^{a}	86.50^{b}	270.00^{c}	860.00^{d}
Zn (mg kg^{-1})	86.50^{a}	42.00^{b}	80.00^{a}	180.00^{c}
Pb (mg kg^{-1})	35.00^{a}	31.56^{a}	67.50^{b}	175.50^{c}
Cu_b (mg kg^{-1})	0.20^{a}	0.20^{a}	0.25^{a}	0.65^{b}
Zn_b (mg kg^{-1})	0.23^{a}	0.25^{a}	0.65^{b}	3.30^{c}
Pb_b (mg kg^{-1})	0.05^{a}	ND	0.25^{b}	0.08^{a}
API	1.30^{a}	1.41^{a}	3.82^{b}	11.36^{c}
Microbial				
Dehydrogenase activity (DHA; µg g^{-1} h^{-1})	83.13^{a}	15.00^{b}	19.11^{c}	17.80^{bc}
Number of 16S rRNA gene copy ($x10^{10}$)	5.38^{a}	2.74^{b}	2.00^{b}	1.82^{b}
Chao1 index of genotype richness (Chao1)	106.50^{a}	87.14^{ab}	75.36^{b}	63.63^{b}
Shannon index of genotype diversity (H')	3.74^{a}	3.43^{b}	3.63^{a}	3.34^{b}
Simpson index of genotype dominance (D)	36.58^{a}	24.50^{b}	34.20^{abc}	24.40^{bc}

Soil microbial properties

The activity of soil dehydrogenase enzymes varied from 83.13 µg g^{-1} h^{-1} in the uncontaminated soil (Chel_1) to 17.8 µg g^{-1} h^{-1} in the most polluted one (Karl_5), indicating a 79% reduction compared to the control (Table 1). Consequently, the dehydrogenase activity tended to decrease along the heavy metal contamination gradient. Many studies reported high sensitivity of DHA to heavy metals and their bioavailable forms. In most of the reported cases, the DHA is very sensitive to high soil concentrations of Cu and Zn (Murata et al., 2005; Wolińska & Stępniewska, 2012; Wiatrowska et al., 2015; Zhang et al., 2008). Resent results were in agreement with our earlier studies, where the rate of inhibition of dehydrogenase activity was up to 88%, and increase with increasing the soil Cu and Zn pollution (Kenarova & Radeva, 2010a; b).

The soils were well abundant of bacteria, whose number varied from 1.82 x 10^{10} (Karl_5) to 5.38 x 10^{10} (Chel_1) 16S rRNA gene copies (Table 1). The bacterial abundance in Karl_5's soil was not dramatically affected by the high HM concentrations, supposing already completed selection followed by a proliferation of HM resistant bacterial species.

The current uncertainties associated with microbial community assessment have, however, given rise to sometimes contrasting observations as to the impact of HM pollution to soil microbial communities. For example, several studies have outlined a lack of microbial community resistance to metal contamination (Gans et al., 2005), while others have demonstrated the opposite trend of extensive HM community resistance (Azarbad et al., 2016). There are also studies reported that HMs affected slightly bacterial abundance, but strongly their diversity (Stan et al., 2011). Our results confirmed the findings of Stan et al. (2011), clearly demonstrating a high bacterial genotype richness (Chao1) and diversity (Shannon and Simpson) in Chel_1, which decreased (significant or insignificant) with increasing the levels of HM contamination in the other sites of interest (Table 1).

Relationships between soil abiotic and microbial properties

Based on the data from Table 1, cluster and principal component analysis (PCA) were conducted to determine the relationships between soil abiotic and microbial properties. The cluster analysis demonstrated the high level of similarity between Chel_1 and Chav_3 (around 78%), both of them forming a cluster of non- to low- polluted arable lands (Fig. 2). The outliers Chel_4 and Karl_5 showed different levels of similarities with the cluster of Chel_1 and Chav_3, expressed by relatively high similarity of Chel_4 (68%) and low similarity of Karl_5 (47%) to the arable lands' environments.

Principal coordination analysis (PCA) was conducted to locate the sites of interest in the ordination space according to their soil physicochemical and microbial properties (Fig. 3). After determining the initial eigenvalues, two principal components were considered, and these components accounted for more than 77.93% of the total variance.

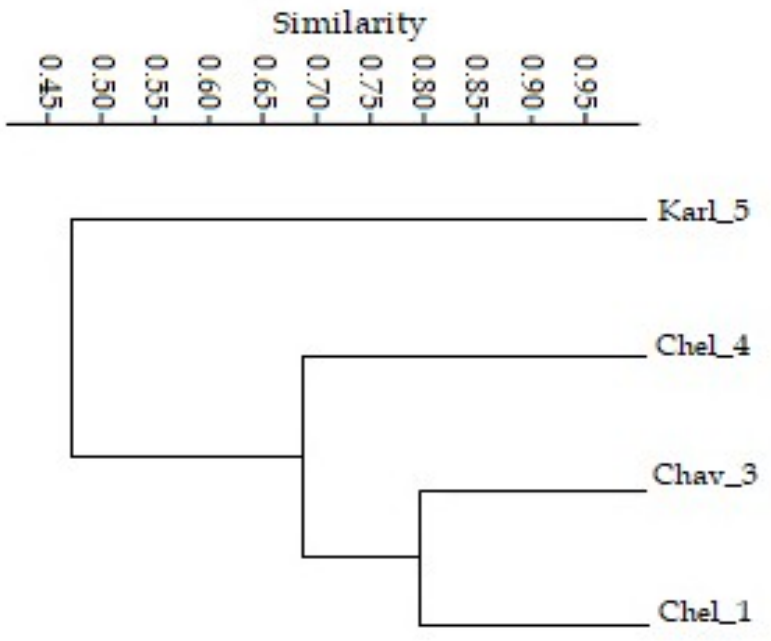

Fig. 2. Cluster dendrogram, representing the similarity among soils based on their physicochemical and microbial properties displayed in Table 1.

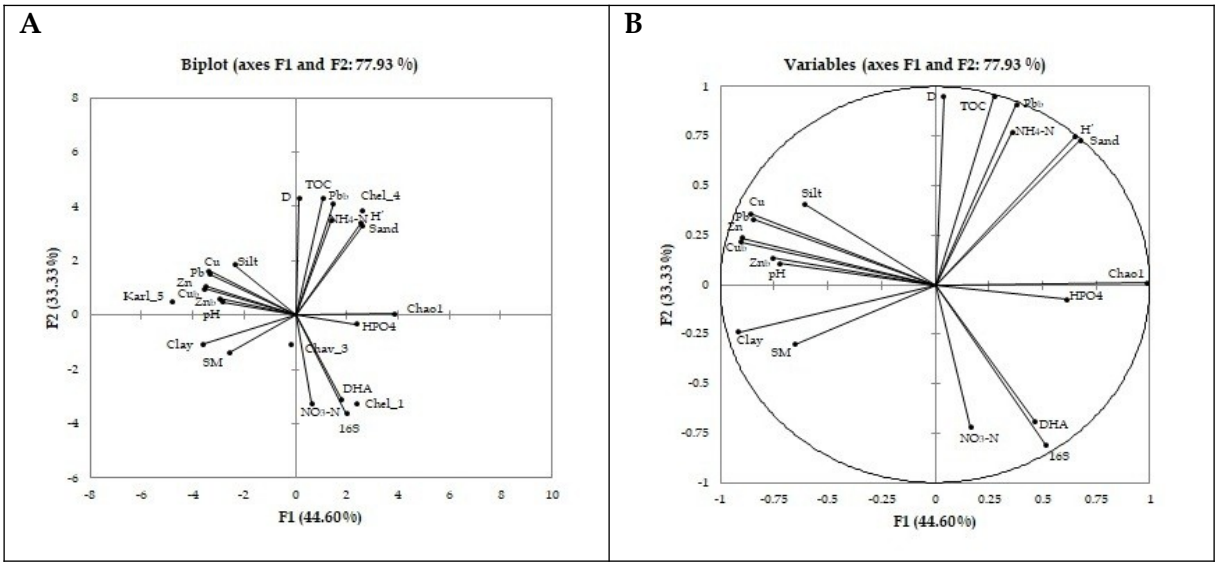

Fig. 3. Principal Component Analysis (PCA). (A) PCA (F1, F2) scores' biplot of sampling sites, based on their soil abiotic and microbial properties, shown in Table 1, and (B) two dimensional (F1, F2) PCA correlation circle, representing the correlation between any two soil variables.

PC ordination confirmed the results of cluster analysis, demonstrating the segregation of Chel_1 and Chav_3 from Chel_4 and Karl_5 along the axis F 1, which loaded 44.6% of the total variability (Fig. 3A). More distinctive for Chel_1 were the high values of dehydrogenase activity, bacterial abundance (16S rRNA gene copies) and genotype richness (Chao1), and soil NO_3-N. Chav_3's trait was strongly linked with edaphic variables such as clay content and soil moisture. Some edaphic (sand, TOC, NH_4-N and bioavailable Pb) and microbial (Shannon and Simpson genotype diversity) properties explained well the features of Chel_4, whereas Karl_5 was well distinguishable from the other sites according to the high content of soil HMs.

PCA analysis showed that DHA correlated significantly and positively with total bacterial abundance (16S rRNA gene copies) and NO_3-N, and positively but insignificantly with the bacterial genotype richness and HPO_4 (Fig. 3B). Significant negative relationships were found between DHA, and soil HMs (except bioavailable Pb), pH and silt (%). The positive correlations of microbial activity, and bacterial abundance and genotype richness supposed a dominant role of bacteria in soil organic matter transformation. Our results confirmed the findings of earlier studies, which revealed a strong significant positive correlation both between soil dehydrogenase and total microorganism count (copiotrophic and oligotrophic bacteria) (Wolinska et al., 2015), and between microbial biomass, and enzyme activities and selected soil properties (Reza et al., 2014). The positive relationships between DHA and soil nutrients (NO_3-N and HPO_4) indicated the nutrient limitation in soils and its crucial effects on soil microbial activity.

DHA correlated negatively with soil total and bioavailable (except Pb) concentrations of HMs. As we pointed above, the negative effects of HMs on microbial activity was evidenced by many authors (Murata et al., 2005; Wolińska & Stępniewska, 2012; Wiatrowska et al., 2015; Zhang et al., 2008), supposing decrease of intensity of many biological processes, including organic matter decomposition and turnover in contaminated soils. Also, the

negative was relationship between DHA and soil pH. In general, the enzyme activity tends to increase with the increasing of soil pH (Błońska, 2010; Moeskops et al., 2010), although the investigations of Wolińska & Stępniewska (2012) indicated a case, where the DHA expressed high activity at pH 5.5 - 5.73, which was significantly inhibited at pH above 5.75. All these facts supposed that the studied soils were inhabited by well adapted to the local pH values acidophilic bacteria, whose metabolic activity was adjusted to the low pH values.

The results of the PCA indicated that bacterial genotype diversity strongly and positively correlated with TOC, sand (%), NH_4-N and bioavailable Pb. Chau et al. (2011) concluded that the light sandy soils are characterized by a larger porous space, which provide more microhabitats that microorganisms can colonized. This in turn increases the potential of many bacterial species to coexist in close proximity without competing for nutrients, and can increase bacterial diversity in soils (Chau et al., 2011). Therefore, the vast spaciousness which has loamy sand soils probably increases the diversity of the indigenous bacterial communities.

It is known that TOC is one of the most important factors affecting soil biodiversity (Fierer, 2017). Soil microorganisms act as "gatekeepers" for the exchange of carbon between the soil and the atmosphere, balancing the accumulation and decomposition of soil organic matter (Malik et al., 2018). Some authors showed positive correlation between the soil organic carbon and the abundance of phyla *Proteobacteria*, *Acidobacteria*, *Firmicutes* and *Verrucomicrobia*, applying the high-throughput sequencing (Trivedi et al., 2016). In our investigations, we evidenced also the dominance of *Proteobacteria*, *Actinobacteria*, *Acidobactria* and *Bacteroidetes* (assessed via 16S rRNA gene retrieval) in the soils of Zlatitsa-Pirdop valley, and changes in the structure of bacterial communities along the heavy metal contamination gradient (unpublished data).

In conclusion, the study demonstrated that: (1) long-term heavy metal contamination has a significant negative effect on soil dehydrogenase activity; (2) DHA correlates significantly and positively with total bacterial abundance and negatively with soil HMs; (3) bacterial genotype diversity is influenced mainly by soil organic matter and soil fraction of sand. This study extends our understanding of microbial activity and its relationships with bacterial diversity and environmental parameters along the heavy metals contamination gradient.

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