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LONG-TERM HEAVY METAL POLLUTION OF SOILS AND ITS IMPACT ON BACTERIAL CARBON METABOLISM

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Abstract

Heavy metal pollution of soils may change their chemical and microbiological status. Changes in the function of decomposer communities may cause disruption in soil nutrient cycling and primary productivity of an ecosystem. In the present study, bacterial capacity to utilize different carbon substrates under heavy metal stress was evaluated by using community level physiological profiling technique and Biolog EcoPlate™ method. Soil samples were taken from the vicinity of mine Chelopech along Cu gradient and co-pollutants Zn and Pb. Soil texture was classified as loam, soil pH was defined as acidic, and soils were determined as well nutrient abundant. Soil concentrations of Cu, Zn and Pb varied in the range of 51–860 mg·kg^{-1}, 44–180 mg·kg^{-1} and 31–175 mg·kg^{-1}, respectively. Both, the capacity of impacted bacterial communities to utilize organic carbon substances and bacterial functional diversity decreased under the heavy metal stress. Bacteria from un-polluted soils preferentially utilized carbohydrates and polymers, whereas the heavy metal stressed bacterial communities preferentially used proteinogenic and non-proteinogenic carboxylic acids. The highest levels of adverse impacts were recorded both at seriously polluted soil, and on the utilization of amines and carbohydrates. Local variability of soil properties might modify the effects of heavy metals. It can be concluded that the EcoPlate™ method can be used to evaluate community functional variability in relation to different levels of heavy metal stress, as statistically significant results have been obtained.

Key words: CLPP, EcoPlate™, heavy metal, mining activities, soil pollution.

Introduction

Soil microorganisms are crucial agents of terrestrial ecosystems promoting the nutritional cycling, soil fertility and energy flow through the decomposer food web (Massenssini et al. 2015, Klimek et al. 2016). Thus, soil microorganisms are strongly susceptible to soil properties, including the effects of various soil pollutants.

Heavy metals (HMs) are the most widespread pollutants, which may signif-

icantly accumulate in soils of industrial and mining areas. Large accumulation may decrease microbial biomass, activity and community structure (Juwarkar et al. 2007, Wang et al. 2007, Chodak et al. 2013, Lenart-Boroń and Boroń 2014, Xie et al. 2016). In this context, microorganisms can play the role of soil quality indicators of the adverse effects of HMs on soil biota. Heterotrophic bacteria are an important part of soil microbial communities closely involved into the organic matter turnover (Massenssini et al. 2015). Generally, heterotrophic bacteria can be described as metabolically heterogeneous (Haferburg and Kothe 2012), and from ecological perspective, determining the changes in their metabolic profiles under HM stress can give valuable information about the capacity of impacted soils to maintain high fertility and primary productivity.

One of the tools appropriate for analysis of bacterial functional diversity is the community level physiological profiling (CLPP) technique (Garland and Millis 1991). Its application is an indirect technique for profiling the changes in carbon metabolism of bacterial communities under pollution stress. CLPP technique uses different microplate systems for bacteria evaluation, but Biolog EcoPlate™ system was created especially for ecological studies, containing 31 naturally disperse carbon substrates (Insam 1997). The advantages of CLPP over other microbial techniques are the simplicity of the protocol and the largely reduced cost. However, some limitations based on its biases as a culturing method were reported – the potential preference of fast growing copiotrophic bacteria (r-strategists of ecological point of view), incubation regime, etc. (Preston-Mafham et al. 2002, Lladó and Baldrian 2017).

The aim of the study was to assess the differences in bacterial functional profiles from HM (Cu, Zn and Pb) polluted soils, using Biolog EcoPlate™ system. Even considering the biases mentioned above, the method should be able to identify shifts in bacterial catabolism under HM stress. Although the method preliminary identifies the activity of copiotrophic bacteria, the results should be indicative for potential impact of HMs on dead organic matter decomposition because the copiotrophs are the key players in the soil carbon cycling.

Materials and Methods

Study area and soil sampling

The study area is in the region of Chelopech mine located in the central-western part of Bulgaria (Fig. 1). The mine has begun operations since 1954 and its exploitation caused soil HM pollution over time (Dinev et al. 2008).

Sampling was done in May 2018 along a gradient of soil Cu concentrations (co-pollutants Zn and Pb) in the vicinity of Chelopech (42.6995° N, 24.0847° E; Chel_1.1, Chel_1.2, Chel_4.1, Chel_6.1, Chel_1.2, Chel_7.1 and Chel_7.2), Chavdar (42.6599° N, 24.0561° E; Chav_3.1 and Chav_3.2) and Karlievo (42.6852° N, 24.1059° E; Karl_5.1 and Karl_5.2) villages. Five subsamples both per site and soil depth [0 – 20 cm (Chel_1.1, Chel_4.1, Chel_6.1, Chel_7.1 and Kar_5.1) and 20 – 40 cm (Chel_1.2, Chel_6.2, Chel_7.2 and Kar_5.2)] were pooled randomly and used for further analyses.

Soil abiotic variables

Soil pH was measured in 0.01 mol L^{-1} $CaCl_2$ (ISO 10390:2005). Soil texture was

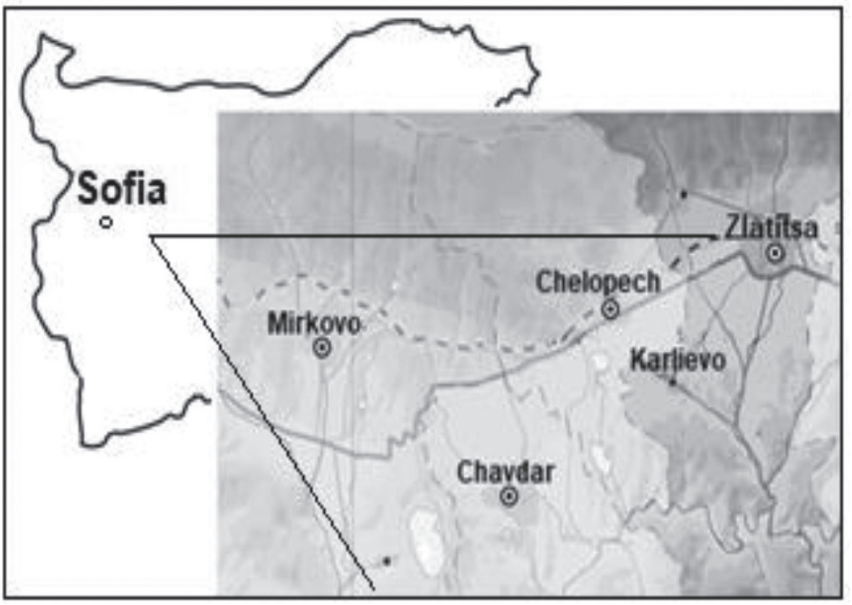

Fig. 1. Sampling locations (Chelopech, Chavdar and Karlievo villages) in the region of Chelopech mine.

determined by Kachinsky method (1958), and the total organic carbon (TOC) – according to Chen et al. (2014). Soil nitrate (NO_3-N) and ammonium (NH_4-N) nitrogen, and inorganic phosphates (P_2O_5) were determined according to the methods of Keeney and Nelson (1982) and Olsen (1982), respectively. Soil moisture (SM) was calculated after oven drying (105 °C). The total concentrations of HMs were analysed 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 0.01 M $CaCl_2$ (bioavailable forms of HMs). Nemerow's pollution index (NPI; Cheng et al. 2007) was calculated, using the total HM soil concentrations, in order to express by single value the level of pollution. The soils were classified as un-polluted at NPI<0.7, and precaution (0.7≤NPI<1.0), slightly (1.0≤NPI<2.0), moderately (2.0≤NPI<3.0) and seriously (3.0≤NPI) polluted, after which were grouped for analysis as follows: un-polluted (Chel_1.1 and Chel_1.2), precaution (Chav_3.1 and Chav_3.2), slightly (Chel_6.1, Chel_6.2, Chel_7.1 and Chel_7.2), moderately (Chel_4.1 and Karl_5.2) and seriously (Karl_5.1) polluted. Different levels of soil pollution were noted as un-polluted (UnP), precaution (PrP), slightly (SlP), moderately (MoP) and seriously (SeP) polluted. The results are given as means and standard errors for the respective pollution level, except SeP, where the means and standard errors were for sampling repeats (*n*=5) of Karl_5.1.

Bacterial functional profiling

Biolog Ecoplates™ system, containing 31 carbon substrates (CSs) in three replicates (Biolog Inc., Hayward CA, USA)

were applied to evaluate the changes occurred in CLPPs under HM stress. The microplates' wells were inoculated with 120 µL bacterial cell suspensions - 1 g soil in 50 mL sterile 0.9 percent NaCl, suspended for 30 min at 240 rpm followed by filtration through 8.0 µm and 3.0 µm pore size membranes. The microplates were incubated at 25 °C in dark. Substrate utilization was monitored every 12 h by measuring absorbance at 590 nm using Microplate Reader LKB 5060-006 and software package DV990 'Win 6'. The measurements of CSs were corrected for background absorbance by subtracting the absorbance of control (water) sample. CSs with corrected optical density (OD) less than 0.25 were considered as non-oxidized and their values were set to zero (Garland 1996). Biolog CSs were grouped according to Weber and Legge (2009) into five carbon guilds (CGs) depending on their chemical moieties: carbohydrates (CH), polymers (Polym), carboxylic acids (CA), amino acids (AA), and amines/amides (Amin). The changes in CLPPs were evaluated calculating the average well colour development (AWCD) according to Garland and Mills (1991) and the maximum utilization rate (MUR) of CG. AWCD and MUR were expressed as CSs' utilization curves (AWCD) and point data at 92 h (MUR). AWCD manifested the total potential of copiotrophic bacteria to metabolize Biolog CSs, whereas MUR indicated bacterial ability to metabolize different guilds of organic substances (CH, Polym, CA, AA and Amin) as both carbon and energy sources.

Data analysis

One-way ANOVA followed by Tukey's test were performed to examine the differences in values of soil properties and bacterial variables. Pearson correlation analysis was passed to identify environmental – bacterial relationships. Above statistics were performed with the package PAST (Hammer et al. 2001) at a level of significance $p<0.05$.

Results and Discussion

Soil environments

Soil textures were classified as loam (UnP, PrP, SlP and MoP) to silt loam (SeP) with pH varying according to SSDS (2017) classification from very strong acidic (MoP) through strong acidic (PrP and SeP) to moderate acidic (UnP and SlP) (Table 1). Soils were well abundant of nutrients with TOC varying from 13.95 $mg \cdot kg^{-1}$ to 26.86 $mg \cdot kg^{-1}$, and the variability of P_2O_5 and inorganic nitrogen (NO_3-N and NH_4-N) ranged from 7.48 $mg \cdot kg^{-1}$ to 11.46 $mg \cdot kg^{-1}$ and from 8.58 $mg \cdot kg^{-1}$ to 49.04 $mg \cdot kg^{-1}$, respectively. The outliers of the general trend of soil nutrient variability were NO_3-N concentrations in UnP and PrP soils.

Cu exceeded Bulgarian maximum permissible concentrations (MPC, 80 $mg \cdot kg^{-1}$) in all soils except UnP, whereas Zn concentrations were under MPC (200 $mg \cdot kg^{-1}$), and Pb was above (60 $mg \cdot kg^{-1}$) in MoP and SeP (Table 2). According to Bulgarian legislation, MPC is the soil HM content ($mg \cdot kg^{-1}$), the exceeding of which under certain conditions leads to soil function disturbance and a risk for environment and human health.

Cu in polluted soils exceeded the background values (UnP) by 1.71 (PrP) – 16.86 (SeP) times. Zn and Pb concentrations were by 0.5–0.8 (PrP – SlP) to 2.0 (SeP) and by 1.0–1.5 (PrP – SlP) to 5.0 (SeP) times higher than the background values, respectively.

Table 1. Soil physico-chemical properties given as means and standard errors (in brackets).

Soil	pH	Sand	Silt	Clay	SM	TOC	P_2O_5	NO_3-N	NH_4-N
		%				mg·kg^{-1}			
UnP	5.70 (1.10)	45.60 (0.00)	37.40 (0.00)	17.00 (0.00)	5.15 (0.15)	13.95 (0.91)	8.92 (6.57)	40.82 (2.13)	2.19 (0.27)
PrP	5.10 (0.20)	45.00 (0.00)	35.80 (0.00)	19.20 (0.00)	7.65 (0.92)	17.44 (7.91)	6.75 (2.17)	45.39 (17.08)	3.66 (1.11)
SlP	5.76 (0.27)	44.90 (0.46)	38.14 (0.03)	16.96 (0.54)	5.43 (0.80)	20.73 (2.08)	7.48 (1.16)	6.60 (2.35)	3.29 (0.57)
MoP	4.80 (0.00)	41.00 (6.92)	40.40 (6.72)	18.60 (0.21)	5.80 (1.06)	26.86 (1.72)	11.46 (1.59)	3.71 (0.95)	4.87 (0.89)
SeP	5.20 (0.06)	31.30 (0.00)	49.80 (0.00)	18.90 (0.00)	7.30 (0.93)	16.86 (0.71)	10.90 (0.64)	8.32 (1.51)	2.60 (0.11)

Table 2. Soil concentrations of total (Cu, Zn and Pb) and bioavailable (Cu^a, Zn^a and Pb^a) forms of heavy metals, and respective NPI.

Soil	Cu	Zn	Pb	Cu^a	Zn^a	Pb^a	NPI
	mg·kg^{-1}						
UnP	51.00 (2.00)	88.25 (1.76)	31.25 (3.76)	0.19 (0.01)	0.13 (0.10)	0.08 (0.02)	0.59 (0.02)
PrP	**89.00** (2.50)	44.50 (2.50)	33.30 (1.70)	0.23 (0.02)	0.22 (0.04)	0.09 (0.04)	0.91 (0.02)
SlP	**134.16** (9.56)	72.80 (8.39)	48.48 (6.31)	0.55 (0.03)	2.48 (0.21)	0.16 (0.04)	1.36 (0.10)
MoP	**251.75** (12.90)	102.50 (15.91)	**91.25** (16.78)	0.38 (0.09)	1.61 (0.67)	0.18 (0.05)	2.48 (0.09)
SeP	**860.00** (9.06)	180.00 (1.37)	**175.50** (3.15)	0.65 (0.04)	3.30 (0.12)	0.08 (0.03)	8.24 (0.07)

Note: HM concentrations that exceeded Bulgarian MPC (Regulation 3, 2008) are given in bold.

Many reports, dedicated to HM soil pollution, evidenced that not the total but the mobile (bioavailable) forms of HMs were toxic for soil organisms (Krishnamurti and Naidu 2002, Rensing and Maier 2003, Gobran and Huang 2005, Brandt et al. 2006, Krishnamurti and Naidu 2008, Xiao et al. 2017). The study found that the bioavailable forms of HMs increased by increasing the levels of soil pollution, being under 0.5 % of total concentrations, except Zn^a which concentrations were 3.4 % (SlP), 4.27 % (MoP), and 1.83 % (SeP) of total. The relation between total and bioavailable forms of HMs was not significant ($p>0.05$), suggesting that not only the level of pollution but also some other local soil peculiarities, such as pH, clay and organic matter content (Krishnamurti and Naidu 2002, Gobran and Huang 2005, Magrisso et al. 2009), might control the concentrations of bioavailable HMs in soils.

Total bacterial catabolic potential

AWCD was used to evaluate bacterial catabolic potential to utilize Biolog carbon sources under increasing levels of soil HM pollution (Fig. 2).

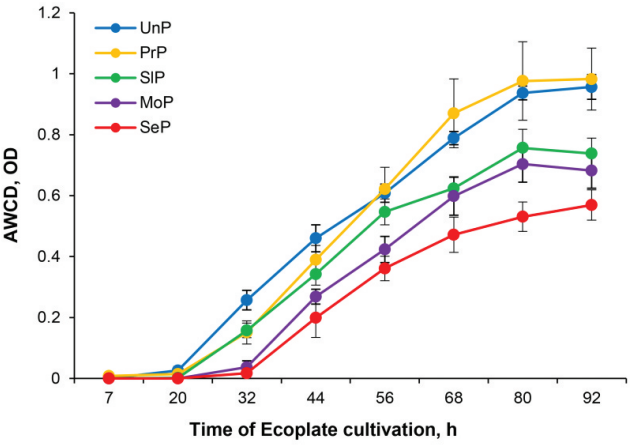

Fig. 2. AWCD of increasing levels of heavy metal polluted and un-polluted soils.

AWCD curves show a general sigmoidal shape, varying in the values of lag-phase (time for adaptation) and slope (maximum utilization rate) in a context of soil HM pollution. HMs affected negatively bacterial catabolic activity, except that of PrP, where the effects were positive. The stimulation effects were manifested by increase of curve slope from 0.121 (UnP) to 0.123 (PrP). For other soils, the curve slopes decreased compared to UnP by 21 % (SlP), 30 % (MoP) and 44 % (SeP). The trend of decrease in maximum utilization rates of Biolog carbon sources was accompanied by the trend of lag-phase increase following the order: 7 h (PrP) < 20 h (UnP and SlP) < 32 h (MoP and SeP). We assumed that the increased bacterial activity in PrP might reflect that Cu and Zn are essential metals for bacterial functioning and in low concentrations act as stimulants for the microbial communities as it was reported by Bruins et al. (2000) and Gadd (2010). At higher HM levels, both essential (Cu and Zn) and nonessential (Pb) metals can damage bacterial cell membranes, alter enzyme specificity, disrupt cellular functions, and damage the structure of DNA. Our results are consistent with the studies of Gryta et al. (2014), Kenarova et al. (2014) and Kuźniar et al. (2018) who all reported decrease of bacterial potential to catabolize carbon sources under metal stress.

Additionally, an interesting phenomenon is clearly manifested on Figure 2 – AWCD curves are very close to each other early on (0–68 h) but diverged later (68–92 h) over the course of time. We suggested that at the beginning of EcoPlates' cultivation, AWCD was dominated by few metal tolerant copiotrophic species, which were positively selected by the high nutrient levels in the wells – phenomenon demonstrated by other authors (Preston-Mafham et al. 2002). After 68 h, the increased curves' divergence might reflect the growth of other species, low abundant, which number/activity inversely correlated with the levels of HM stress. The manner of delayed curve divergence on Figure 2 confirmed the above mentioned suggestion, indicating the highest bacterial functional (metabolic) diversity in UnP and PrP and the lowest one in SeP. Similar phenomenon of late curve divergence was evidenced by Muñiz et al. (2014) and Yuangen et al. (2006) in their experiments, studying the HM effects on bacterial functional diversity.

Kinetics of carbon guilds' utilization

To further compare the catabolic diversity among different levels of soil HM pollution, Biolog carbon sources were grouped into carbon guilds. The ratio of CGs' utilization rates in AWCD and their kinetic curves are shown on figures 3a and 3b-f, respectively.

UnP's bacterial metabolism was well balanced (Fig. 3a), and the share of CGs' utilization in AWCD value (point data at 92 h) slightly decreased in the order: Amin > Polym ≥ CH > CA ≥ AA. CGs' utilization over time formed sigmoidal curves, which differed each other by the longevity of lag-phase and the value of curve slope (Fig. 3b-f). In general, HM stress caused extinction of lag-phase (except at CH for PrP – MoP) and decrease into the curve slopes (except AA and CA for PrP).

CGs' utilization under stress delayed

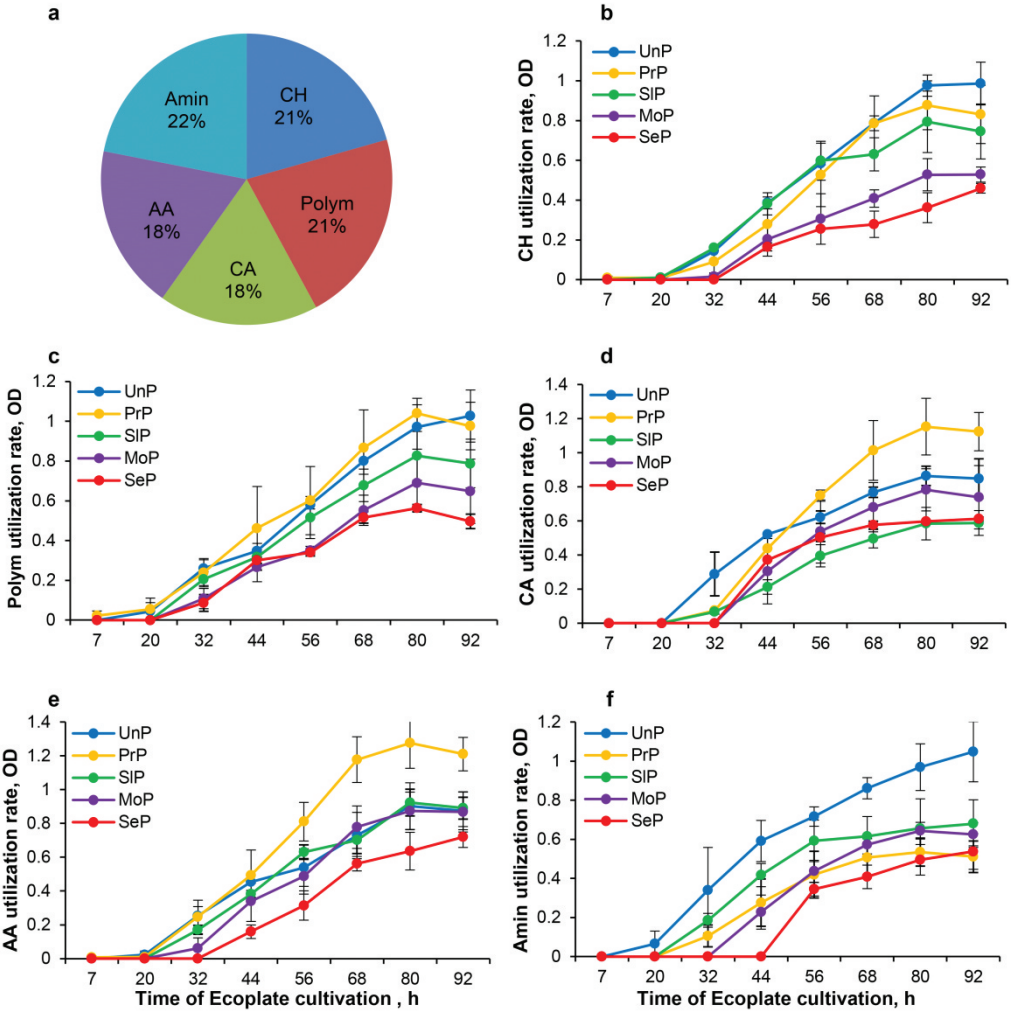

Fig. 3. Ratio of CGs' utilization rates in AWCD (a), and kinetic curves.

commonly by 12 and more hours. For example, the utilization of AA and Amin at SeP soil delayed by 24 h and 36 h, respectively, compared to UnP. A twenty-four-hour delay was recorded also for Amin utilization at MoP soils. On the other hand, HM stress shortened the lag-phase of CH sigmoidal curves of PrP, SlP and MoP soils. This fact indicated that the enzymes involved into carbohydrate catabolism in HM impacted cells were already synthesized, probably to gain more energy for stress overcoming. The highest level of soil pollution (SeP) inhibited this process.

It was recorded that bacterial catabolism misbalanced under HM stress. The curve slopes (MUR) under soil pollution decreased and the ratio between the utilization rates of CGs within AWCD changed. The curve slopes of HM impacted CGs' utilization decreased in the order: PrP: AA > CA > Polym > CH > Amin; SlP: AA > Polym > CH ≥ Amin > CA; MoP: AA > CA > Polym > Amin > CH; SeP: CA > AA > Polym > Amin > CH. Under HM impact, the most preferable CGs were AA and CA and the less preferable - Amin and CH. Some exception was evidenced for SlP soils, probably due to the modulating effects of some local soil peculiarities.

Similar shifts in metabolic profiles were evidenced in earlier experiments (Yuangen et al. 2006, Kenarova et al. 2014, Muñiz et al. 2014, Kuźniar et al. 2018), where it was found that AA was the most intensively metabolized CG by HM stressed soil bacteria, whereas CH and Polym were preferred carbon sources by un-impacted ones. What was the reason of bacterial catabolic shift under stress was not clear. We suggested that HM stress induced detoxification mechanisms in microbial and plant cells involving AA synthesis – macromolecules that could later be metabolized by the soil microbiota, which was demonstrated in the reports of Hall (2002) and Sharma and Dietz (2006).

The highest decrease of MUR was detected for SeP. Compared to UnP, MUR of CA and AA decreased by 30 % each; Polym, Amin and CH decreased by 43 %, 56 % and 60 %, respectively.

However, some stimulation effects compared to UnP were observed – for example, MUR of AA, CA and Polym at PrP increased by 42 %, 23 % and 4 %, respectively.

The late kinetic curve divergence was observed almost for all CGs (Fig. 3b-f), and the mode of divergence corresponded to the respective CG. ANOVA analysis of kinetic curves (covered only the period of curve divergence: 68–92 h) confirmed the significance of divergence pattern. Because we assumed that the late kinetic curve divergence reflected the functional diversity of soil bacterial communities growing on the respective CG, we could notice that: 1) functional diversity of Amin utilizing bacteria was the highest at UnP and it was reduced even by the lowest HM pollution level ($p<0.001$); 2) HM pollution stimulated the functional diversity of bacteria on AA (PrP; $p=0.000$) and CA (PrP and SlP; $p<0.001$), and inhibited that at SeP (AA: $p=0.023$ and CA: $p=0.002$); 3) CH and Polym compared to UnP supported less functionally diverse communities at SlP (CH; $p=0.045$), MoP (CH and Polym; $p<0.007$) and SeP (CH and Polym; $p<0.001$).

Conclusion

Chelopech mining activities caused HM (Cu, Zn and Pb) accumulation in soils, being classified according to NPI, as precau-

tion to seriously polluted ones. HM pollution affected negatively the catabolic activity of impacted bacterial communities, decreasing their potential to metabolize soil organic carbon matter. HM pollution decreased the functional diversity of bacterial communities capable to utilize Amin (PrP – SeP), and CH and Polym (SIP – SeP). Low (PrP – SIP) and high (MoP – SeP) levels of HM pollution increased and decreased, respectively, the functional diversity of bacteria capable to utilize CA and AA. The above results conclude the suitability of Biolog EcoPlate™ method for studies on soil bacterial carbon metabolism under HM pollution.

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