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Microbiota as an indicator of the environmental burden of mined-out sites

K Boturova

Department of Environmental Engineering, VŠB – Technical University of Ostrava, 17. listopadu 15/2172, 70833 Ostrava – Poruba, Czech Republic

Abstract. The article focuses on the characteristics of the microbial community in the post-mining territory of Poproč (Slovak Republic), where polymetallic ore was mined in the past. The investigated waste substrates are characterized by the still high presence of antimony, arsenate, lead and other toxic and potentially toxic metals and semimetals. Microbial activity in post-mining substrates was established on the basis of the presence of functional microbial biomass by fumigation-extraction method. The results are complemented by additional ecosystem parameters, in particular, the representation of individual cultivable groups of microorganisms. The activity of the microbial community is in direct interaction with the post-mining environmental burden and can, therefore, be considered as an indicator of the revitalization of the *in situ* environment.

1. Introduction

Soil is a complex environment with a very diverse composition of organisms from microorganisms to macroorganisms; in this complex ecosystem, each organism performs its important role. It has been proven that the appropriate indicator of the state of soil revitalization at post-mining sites is just microorganisms [1, 2, 3, 4, 5, 6]. Their diversity, vitality and active physiology affect soil quality and thus many other aspects of the anthropogenic contaminated environment – not only the soil ecosystem but also the aquatic and atmospheric environments, which are closely related to the terrestrial environment. Also, the extensive degradation and resistance capabilities of microbes are increasingly used in bioremediation processes to remove all sorts of pollutants from contaminated environments. [7, 8, 9, 10, 11, 12]. Knowledge of the diversity of the environment is particularly important – the correct identification and taxonomic inclusion of microorganisms can derive certain assumptions, properties and biogeochemical activity of these biots, which is important in biotransformation processes in the environment. [13, 14, 15]. The biochemical properties of microbes can be successfully further exploited, based on the microbiology of the majority of modern biotechnological productions of the present period [16, 17, 18, 19, 20].

2. Materials and methods

Site characteristics

The Poproč antimony deposits with adjacent extractive waste sludge lagoons are located in the valley of Petrov, northeast of the village of Poproč in the eastern part of the Slovak Republic. There is an old environmental burden. It is a site of already closed mine workings, which belonged to very important sites of antimony ores in Slovakia until 1965. The mined ore had a polymetallic character, and in addition to a significant proportion of antimony, it also contained other toxic and potentially toxic metals and half-metals (e.g. As, Pb, Cu, Fe, Zn, Co, Cd, Ni, and Au). The ore was further processed by the



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flotation method, which produced waste, which was deposited into three different sludge lagoons situated above the municipality of Poproč [9]. Nowadays, the sludge lagoons are undergoing recultivation and revitalization of the landscape in the form of new green plantings and restoration of ecosystem function.

Sampling of soil substrate and determination of basic characteristics

Samples of potable substrates Poproč have been taken from different places to include all three of the sludge lagoons. Soil samples from a depth of 0 – 20 cm were taken using a sterile soil auger (in the amount of approx. 2 kg) and mechanically removed larger parts of plants. The samples were subsequently enclosed into sterile plastic bags which were kept cool and immediately taken to the laboratory to other analyses. After arriving in the laboratory, the samples were processed by the standard procedure: homogenizing, quartering and sieving to fraction < 2 mm (according to norm ČSN ISO 3310-1). Part of the samples was used for basic physicochemical analyses, and another part of the samples was used in the determination of microbial characteristics of samples (dilution plate method, fumigation extraction method and diversity of microorganisms using biochemical identification system BIOLOG). The accredited laboratory of MORAVIA s. r. o. in Studénka, the Czech Republic, carried out the chemical and physicochemical laboratory analyses for soil reaction. Samples of soil substrates of the sludge lagoon were determined by the SOP 44 method (ČSN EN 15933) for pH active, and for pH_{KCl}. SOP 06 method according to the standard ČSN ISO 10390 (2005) was used. For total organic carbon, the method, according to SOP 56 (ČSN EN 15936) was used. The dry matter in the samples was determined using the Gravimetric method according to the standard ČSN ISO 11465:1993. The contents of the elements were determined using an X-Ray Fluorescence Spectrometer (WD-XRF S8 Tiger, Bruker Co, USA) at the Centre of Advanced Innovative Technologies at VŠB – Technical University of Ostrava.

Isolation, cultivation and identification bacterial isolates

Bacteria were isolated by cultivation methods on nutrient media using the dilution plate method [22] on tryptone soya agar (M 290, HiMedia Laboratories Ltd., Mumbai, India) and cultivated at 30 °C for 48 h. All morphological varieties of colonies were selected and inoculated into the original nutrient medium to obtain pure bacterial strains. Pure cultures were identified morphologically and according to their characteristics. Biodiversity of microorganisms was determined according to the BIOLOG system.

Determination of microbial biomass

Chloroform fumigation extraction method was performed according to ISO norm 14240-2 1997. The samples were moistened to 44% WHC (maximum water-holding capacity) and divided into 2 parts: NF (which were not exposed to fumigation) and F (exposed to 24-hour fumigation). Each part of the method was performed in three iterations. The NF samples were adjusted 0.5 M of potassium sulphate, and subsequently, the samples were shaken on a horizontal shaker (175 oscillations per minute) for 30 min, then the samples were filtered. Subsequently, the NF samples were closed at 4 °C and left until the carbon determination. F samples were inserted into the exicator, where they were exposed to fumigation with chloroform (vacuum of 180 millibars). The samples were then left in the exicator in the dark at laboratory temperature for 24 hours. The next day, the remaining chloroform was extracted (in six repetitions). Subsequently, to fumigation samples was added 0.5 M potassium sulphate, the samples were shaken and filtered (see Figure 1).

The samples were modified with a chromosulfuric mixture: they were mixed with K₂Cr₂O₇, H₃PO₄ and H₂SO₄ and burned in a drying oven at 125 °C for 45 minutes. After the samples cooled, titration with an indicator (phenylanthranilic acid) was performed. Finally, the samples with the indicator were titration with Mohr's salt. Titration was terminated by a colour reaction.

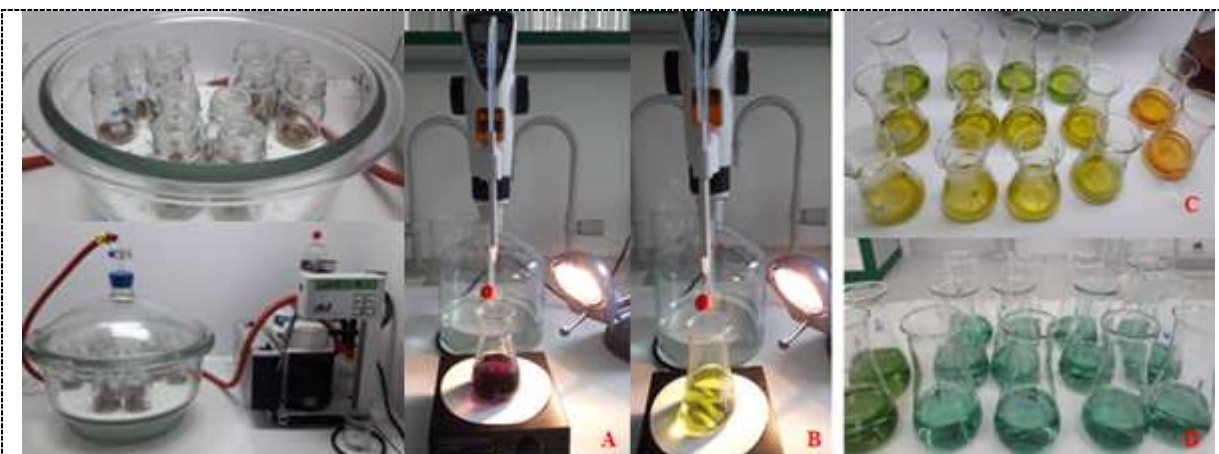


Figure 1. Fumigation with chloroform in the excicator and determination of the carbon in the extract; A – the violet colour of the solution before completion of titration, B – end of the titration, C – discolouration of the samples without fumigations phase, D – fumigation phase colouring (photo by author, 2019).

3. Results and discussion

Microbial characteristics

The combination of parameters describing the state of the microbial community will enable the general extrapolation of the results to the state of the environment, in which microorganisms serve as indicators of anthropogenic pollution and thus the state of soil quality, its revitalization [23, 24]. One advantage of the combination of microbial parameters is the early warning [25] when this ability is attributed to the high sensitivity of soil microorganisms, their interactions with the environment at a microscopic level and the rapid reaction to stress [26, 27]. Nevertheless, for the correct evaluation of soil quality, in addition to the combination of various microbial parameters, the other parameters of biological, physical, chemical or physico-chemical are to be investigated together with microbial parameters [28, 29, 30]. Based on this knowledge, the soil type and other basic characteristics of the substrate (Figure 2) have been determined.

POP 1	POP 2	POP 3
Type: sandy loam soil Depth: 0 - 20 cm $\text{pH}_{\text{H}_2\text{O}}$ 5.8 pH_{KCl} 5.3 TOC 0.20 % Dry matter: 87.13 %	Type: sandy soil Depth: 0 - 20 cm $\text{pH}_{\text{H}_2\text{O}}$ 5.1 pH_{KCl} 4.4 TOC 0.15 % Dry matter: 88.08 %	Type: clay soil Depth: 0 - 15 cm $\text{pH}_{\text{H}_2\text{O}}$ 4.5 pH_{KCl} 4.0 TOC 0.18 % Dry matter: 80.05 %
As 5266 mg/kg Sb 16455 mg/kg Pb 813 mg/kg	As 3303 mg/kg Sb 7711 mg/kg Pb 865 mg/kg	As 2562 mg/kg Sb 7401 mg/kg Pb 575 mg/kg

Figure 2. Selected characteristic and content of potentially toxic elements in the investigated samples of Technosol Poproč (average values from 5 sampling points from each sludge lagoon).

Table 1. Isolated bacterial strains.

Taxonomic group	Species affiliation	Place of collection in the pond			Probability	Similarity
		POP 1	POP 2	POP 3		
Firmicutes	<i>Bacillus alcalophilus</i>	+	–	+	96	0.562
	<i>Bacillus amyloliquefaciens B</i>	+	+	+	100	0.728
	<i>Bacillus atrophaeus/subtilis B</i>	+	+	–	93	0.617
	<i>Bacillus cereus</i>	+	–	–	87	0.749
	<i>Bacillus fastidiosus</i>	+	+	–	83	0.675
	<i>Bacillus licheniformis</i>	+	+	+	86	0.531
	<i>Bacillus marococanus</i>	–	–	+	–	0.414
	<i>Bacillus megaterium</i>	+	–	+	87	0.749
	<i>Bacillus megaterium A</i>	+	+	+	94	0.796
	<i>Bacillus mycides</i>	+	+	–	98	0.745
	<i>Bacillus pumilus C</i>	+	+	+	100	0.656
	<i>Bacillus simplex</i>	+	–	+	100	0.738
	<i>Paenibacillus illinoisensis</i>	–	–	+	87	0.542
	<i>Paenibacillus macerans,</i>	–	–	+	96	0.562
	<i>Paenibacillus pabuli</i>	+	–	–	100	0.771
	<i>Paenibacillus polymyxa</i>	+	–	+	100	0.793
	<i>Staphylococcus warneri</i>	+	–	–	95	0.549
Actinobacteria	<i>Acinetobacter radioresistens</i>	–	–	+	95	0.692
	<i>Arthrobacter histidinolovorans</i>	+	+	+	99	0.610
	<i>Arthrobacter ilicis</i>	+	–	+	96	0.582
	<i>Brevibacterium otitidis</i>	–	–	+	86	0.531
	<i>Cellulomonas hominis</i>	–	–	+	88	0.596
	<i>Micrococcus luteus E</i>	+	+	+	94	0.646
	<i>Micrococcus yunnanensis</i>	+	–	+	91	0.704
Bacteroidetes	<i>Flavobacterium sasangense sp.</i>	–	–	+	89	0.523

<i>β-Proteobacteria</i>	<i>Paraburkholderia caledonica</i>	+	+	–	91	0.549
<i>γ-Proteobacteria</i>	<i>Pseudomonas putida</i>	+	+	+	90	0.697

All the substrates studied (Figure 2) had a medium acidic environment (values of $\text{pH}_{\text{H}_2\text{O}}$ 4.5 až 5.8 and the values of pH_{KCl} 4.0 až 5.3). Total Organic Carbon ranged from 0.15% to 0.20% in the samples analysed. The content of all observed chemical elements highly exceeded the limit values. The monitored substrates are highly contaminated with toxic metals, mainly arsenic and antimony, the highest arsenical and antimony content ($\text{As} \geq 5266 \text{ mg/kg}$, $\text{Sb} \geq 16455 \text{ mg/kg}$) was detected in POP 1 samples taken from 0 – 20 cm in depth. Increased content of lead and other toxic and potentially toxic metals and half-metals was also detected in all samples.

The basic microbial parameters were determined according to the criteria of Nielsen and Winding (2002) [28]: measurement of abundance and biodiversity of soil microorganisms, measurement of microbial biomass and measurement of total organic carbon and identification of key species of autochthonous microorganisms.

The diversity of isolated microorganisms was analysed by the system BIOLOG. One of the critical parameters for the characteristic of microbes is the identification of key types of autochthonous microorganisms, which can then be used in the recultivation or revitalization of the area affected by mining [30]. The most numerous was the *Firmicutes* strain with *Bacillus*, which occurred in 44% of the entire cultivable ingredient. Table 1 contains the previously identified bacteria of the genus *Bacillus*, in which 12 species have been identified. The species bacterial diversity in sludge lagoons Poproč suggests their capacity to adapt and prosper in their own ecological niche.

Values of microbial biomass (C_{mic}) determined by fumigation extraction method [31] were very similar in samples from sludge lagoon 1 (POP 1) and sludge lagoon 2 (POP 2), average sample value ranges from $550.36 \mu\text{g C}$ to $615.63 \mu\text{g C}$ per gram of substrate (Table 2). The highest value ($656.63 \mu\text{g C}$ per gram of substrate) was observed in the sludge lagoon 1 (POP 1), where it was once higher than the third (Figure 3). This is probably due to a layer of moss and low vegetation typical of this locality, which created more suitable microclimatic conditions for the existence of soil microorganisms (from POP 1 was isolated 20 species of bacteria, of which 11 species of the genus *Bacillus* from a total of 12 isolated). The activity of the soil microbial community can also be closely combined with soil moisture, soil temperature and, in particular, the biological usability of organic substances [32, 33, 34]. The lowest values of microbial biomass (POP 3 – $309.19 \mu\text{g C}$ per gram of substrate) showed limited availability of organic substrates and their degradation in the type of clay soil, but also on the slower intake of nutrients delivered through biogeochemical cycles [35].

Table 2. Result values microbial biomass C_{mic} ($\mu\text{g CO}_2\text{-C.gdry matter}^{-1}.\text{h}^{-1}$).

	POP 1	POP 2	POP 3
Number of samples	5	5	5
WHC max [ml/g]	0.4519	0.4982	0.7127
Addition of water for 44 % WHC [ml/g]	0.0482	0.0726	0.0134
Arithmetic mean [$\mu\text{g/g}$]	615.36	550.36	322.29
Max [$\mu\text{g/g}$]	656.63	621.25	324.56
Min [$\mu\text{g/g}$]	535.55	515.13	309.19
Median [$\mu\text{g/g}$]	634.64	532.23	319.58

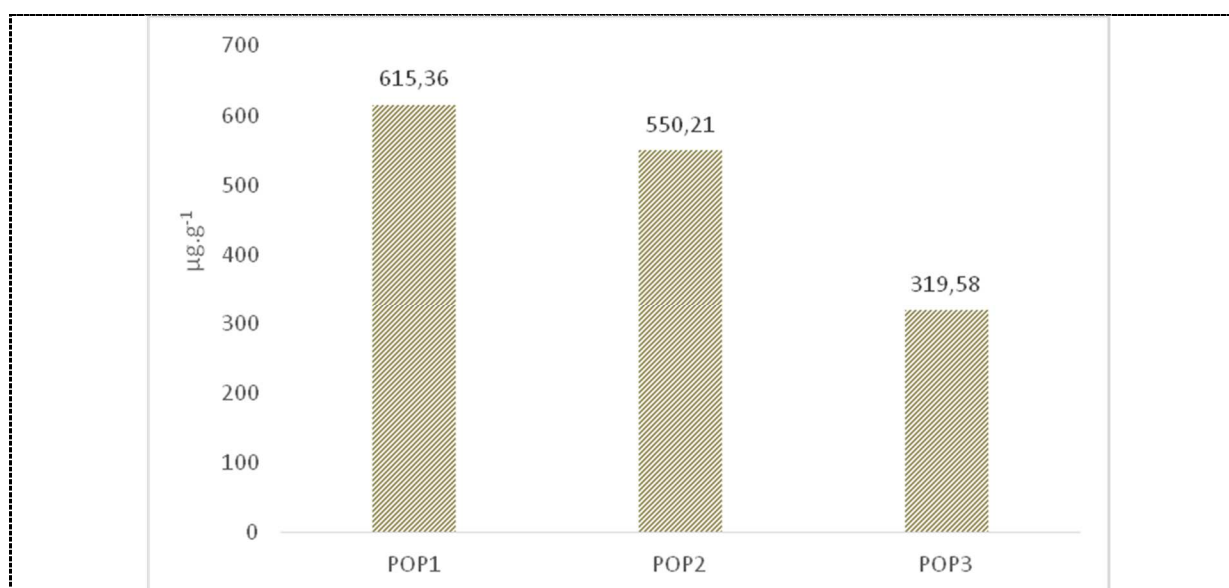


Figure 3. Soil microbial biomass values (C_{mic}) estimated by fumigation extraction in the investigated samples of sludge lagoons Poproč.

The average value of basal respiration rate increased from the POP 3 sample ($10.91 \mu g/g$ substrate) to POP 1 ($23.81 \mu g/g$ substrate). However, in Sample 1, the highest post-titration breathing value was found (Figure 4). The relatively low values of basal respiration of microorganisms in all samples were probably due to high levels of potentially toxic semi-metals (especially As and Sb). Such contents of metals resulted in decreasing of carbon dioxide production by microorganisms [36]. Soil microbial indicators such as microbial biomass or basal respiration may serve as one of the environmental pollution indicators [37, 38].

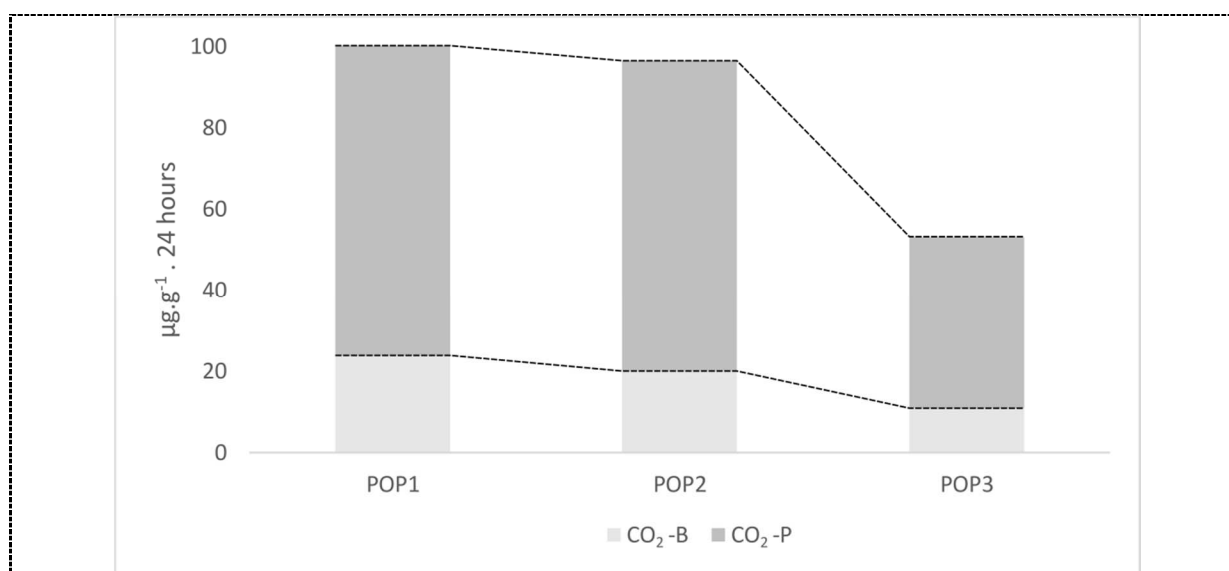


Figure 4. Basal and potential respiration ($CO_2 - B$; $CO_2 - P$) determined by quantifying the carbon dioxide released in the process of microbial activity in the investigated samples of sludge lagoon Poproč from each sludge lagoons).

Many scientific studies [39, 40, 41] demonstrate that certain microorganisms are able to survive in soils and technogenic sediments contaminated by high concentrations of toxic metals and semi-metals. An example is the *Firmicutes* strain with the identified genus *Bacillus*, which occurred in sludge substrates with the highest observed abundances (62.96%). Bacteria of the genus *Bacillus* are known for their

physiological ability to produce a wide range of metabolites – enzymes, organic acids, vitamins and toxins [42, 43]. In their biological cycles, there are also several basic mechanisms of resistance to arsenic and antimony, for example, regulating proteins encoded with *aioA* genes, *aioB* and others for the Sb-oxidase [43], the *ArsB* gene system for expression As-resistance [39] and many other mechanisms. These systems are often based on the bacterial extracellular barrier based on the presence of surface exopolymers composed of glycides, polysaccharide, nucleic and fatty acids, which complement other processes of reduction of metal ions by means of efflux, extracellular sequestration, intracellular sequestration and active transport [44].

In addition to the above mechanisms of natural resistance, many production processes of metabolites can be further improved and supported by molecular genetic interventions in the bacterial genome, including those areas that provide genetically conditioned adaptation to increased arsenic and antimony in the environment [42].

Also in the case of observed bacterial isolates from the site of Poproč, it was confirmed by several representatives of autochthonous microbes that *Ars*-operon, *AioB*-operon and other systems of genetic resistance to As and Sb are present in the genome of isolated autochthonous bacteria [24]. Since these genes can be subjected to horizontal transmission, it can be assumed that other isolated bacterial autochthonous microbes *in situ* also contain the listed components of the genome.

The ability to withstand long-term exposure to increased concentrations of toxic metals and half-metals has been previously supported in bacteria *Bacillus* by a number of studies [45, 46, 47]. The very high presence of arsenic (≥ 5266 mg/kg) and antimony (≥ 16455 mg/kg) in the sludge substrate resulted in the selection of specifically resistant microorganisms for which interesting phenotypic properties of the BIOLOG system were confirmed. Also due to the physiological capacities of the spores, the *Bacillus* genus can well survive this strong selection pressure of metal-contaminated substrates and can, therefore, be considered as the general representative of the indicator group of highly adaptable microorganisms for metal contamination [27, 48, 49, 50]. The fact that the genus *Bacillus* has been confirmed as prevalent in the cultivable part of the detected autochthonous microbes, it can also be assumed to have a crucial role in the biological biotransformation of metals at the same time as the gradual changes in the geochemical conditions in soils after mining and industrial activities.

4. Conclusion

The soil represents a vast reservoir of various groups of microorganisms, although it is generally stated that the proportion of the cultivable ingredient is approximately only 1% of the total amount of bacteria in this environment [36]. For this reason, it is essential to pay attention to the isolation of new, yet uncultivated, bacterial microorganisms often bearing new genes coding the unusual forms of their products. These new isolates can find their potential use in various fields of applied microbiology, for example in the production of biologically active substances active in bioremediation processes and in many others, in which the participation of microorganisms or their products is an indispensable part of biotechnological processes.

Of the available analyses of the condition of highly contaminated locality Poproč (Slovakia), the strain with the highest abundance is formed by *Firmicutes*, whose biochemical properties have been verified by the BIOLOG system. It has been confirmed that the most widely represented *Bacillus* genus is very variable and adaptive and can, therefore, adapt well to substrates contaminated with toxic metal ions. Specific physiological profiles of identified species illustrate the possibility of rapid physiological adaptation to adverse physical, chemical and biological environmental conditions. Confirmation of the presence of predominant representatives of the genus *Bacillus* in sludge substrates after extraction of arsenic, antimony and lead means a direct link to the process of revitalizing the landscape influenced by mining and restoring the original biological function of the environment.

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