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5. ECOLOGY AND ENVIRONMENTAL PROTECTION

EVALUATION OF SOIL MICROBIAL COMMUNITY PARAMETERS IN CORRELATION WITH METAL POLLUTION GRADIENT

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Abstract

This report provides an information about specific physicochemical site description, focusing on determining the microbial content of the sampled lead and copper polluted soil of the site location, its quality. It contains reasoning for the analysis, methodological part with bibliographic studies of the location given. Available on–site bacteria in the top soil layer of 0 – 0.5m tested for the microbiological content analysis, using latest Next Generation Sequencing (NGS) technologies – "Illumina MISEQ" sequencer with read length of 250 cycles both ways on a 457 base pare DNA amplicon.

Since microorganisms are sensitive to low pollution concentration levels, this implies, that they could serve as more accurate pollution indicators. Thus, five boreholes were made to sample the soil at the research site, which has been located as a quay between the city and meadow landscape. Almost each soil sample has different metal concentration.

According to the NGS results Acidobacteria (up to 26%), Proteobacteria (up to 20%), Actinobacteria (up to 15%) and Firmicutes (up to 12%) bacteria phyla are dominant in the sampled soil. Also found Nitrospirae bacteria, which is a globally distributed group of nitrite oxidizers, playing important role in nitrogen cycle. Comparing the results of this analysis and the review made in the annex 1 – a few similar genera were found. One of the reasons to the fact, that the most abundant phylum in given soil is Acidobacteria – is that this phylum was also observed to be found in metal–contaminated soils and sediments according to Thrash J. and Coates J., 2015, however in current situation the same correlation has not been found.

Next steps for the project: AMR (anti metal resistance) analysis, binding the correlation between the concentration of the pollutant and its impact on the microbial population.

***Key words**: Soil microorganisms, Next Generation Sequencing, Lead/Cooper pollution, environmental risk assessment.*

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As the company prefers to keep clients confidentially – official names, towns, and coordinates in which the example assessment was conducted will never be mentioned in this report nor the contacts of the people involved on specific projects.

Introduction

Nowadays in our rapidly developing world, we are exposed to different types of human and environmental risks. World Health Organization stated in 2005: "environmental factors being the root cause of a significant disease burden, especially in developing countries. According to the "Assessment of human health and environmental risks of new developments in modern biotechnology" – policy report of National Institute for Public Health and the Environment, 2018: "in half of investigated new applications, the risk assessment methods may no longer be adequate, or having a lack of information available to effectively assess risks". Therefore, we have to be ready for the use of many new applications, because the current risk assessment method may not be the best choice, remaining unclear whether an assessment method is particularly usable, leaving possible risk exposures.

According to Yergeau E. et. al., 2012, Dian C., 2018, Milosevic N. et al., 2010: "Microorganisms form the base of the food chain in ecosystems, they are key players in biogeochemical cycles – degradation and decomposition of organic and inorganic compounds, primary production and nutrient cycling, climate regulation. Consequently, their pollution response can disproportionate impacts on ecosystem functioning and biodiversity in total. Soils, polluted with heavy metals (further referred to as "Me") – possess negative effect on the total soil respiration rate and enzymes activity. Once these pollutants are in the soil: "the primary impact is the amount of soil bacteria, actinomycetes, fungi and other microbial populations, altering not only their qualitative, but also quantitative community parameters". Evidently, it is crucial to understand microbial responses (changes in community composition, its functioning, etc.), showing that the environment is to be protected or reclaimed. "These microorganisms, however, are sensitive to low concentrations of the pollutant, and it is conceivable that they could serve as more accurate indicators, be used as an assessment tool" (Dian C., 2018).

Based on this, the aim of current internship is to make an investigation in the sphere, defining an impact of specific type of pollutants on the quality of microbiological communities. For the future work, the analysis of current microbial responses towards the exposure with different hazard concentration levels will be held. Overall future use and prospects of the implementation of such a "bio–indication" approach in the risk assessment procedures will bring the worldwide advanced environmental legislation standards on a new level.

The output of this work will be a progress report, which will focus on physicochemical site description, determining the microbial content of the sampled soil of the site location, its quality. It will contain reasoning for the analysis, methodological part, bibliographic studies of the selected location, with following results outcome, interpretation of scientific data, topic discussions, conclusion and recommendations.

Unit 1
General information and study framework

SWECO AB (originally "Swedish Consultants") is a European engineering consultancy company, active in the fields of construction, architecture, and environmental engineering. It was founded by Gunnar NORDSTRÖM in 1997, with the headquarter KUNGSHOLMEN in Stockholm, Sweden.

As of 2015, SWECO has about 14,500 employees, revenue of about 1.8 billion euro and presence in: Sweden, Norway, Finland, Estonia, Denmark, Netherlands, Germany, the Czech Republic, Poland, Lithuania, the United Kingdom, Belgium, Bulgaria and Turkey. SWECO plans and designs the communities and cities of the future. The results of the work are sustainable buildings, efficient infrastructure and access to clean water and safe environment.

The company is listed at the stock exchange in Stockholm and has its historical roots in a number of companies, now merged, such as AB VATTENBYGGNADSBYRÅN, VIAK AB, FFNS AB and GRONTMIJ.

Current internship topic was based on the departments' research interest. With the willing of future results to be used for the implementation as reference standards for soil risk assessment, including it as a particular service for SWECO clients.

All the necessary data and equipment was provided to perform the internship, all the scheduling, duties and responsibilities were according to the internship agreement.

1.1. Problem analysis and itsobjective

Microorganisms are sensitive to low concentrations of the pollutant. This implies, that they could serve as more accurate pollution indicators (Yergeau E. et.

al., 2012). By means of the chronic and/or acute changes in the local microbial community composition, functions and pollutant resistance, particular response will be used as indication of pollution presence, alongside other usual environmental standards, such as AW2000 in Netherlands.

The objective of this research is to investigate the specific site location, polluted with cooper and lead, using types of available on–site bacteria in the top soil layer of 0 – 0.5m for the microbiological analysis, such as soil content, using latest Next Generation Sequencing technologies, interpreting the scientific data. The further future works, continuing the project: Anti–Metal Resistance patterns, binding the correlation between the concentration of the pollutant and its impact on the microbial population.

1.2. Relevance of the research

A number of studies published over the past decade highlighted the role of microbial diversity for ecosystem functioning, including potential consequences of reduced diversity to the stability of microbial communities (Bell T. et al., 2005), one of such studies was made by Dian C., 2018, where it is said: "Due to the persistent, non–biodegradable and sometimes toxic nature of heavy metals in the soil, current pollution issue has attracted increasing attention of researchers. Since microbes are more sensitive to environmental stress than macro–organisms in soil ecosystems, they can also reflect changes in soil environment as early as possible and are therefore considered to be sensitive indicators in the soil".

Type of the residential location and interaction of specific pollutants, such as lead and cooper in different concentrations available on site, provides specificity of current research.

The chosen site will be sanitized in the near future, so an additional benefit would be – assessing an efficiency of the chosen remediation strategy.

Unit 2
Context of the research investigation

2.1. Literature review and site description

Bibliographic studies were conducted from peer review articles and previous investigation conclusions of expert commissions on the site – documents of SWECO projects database.

The research site has been located as a quay between the city and meadow landscape from at least 1815. In the 1970s a residential area was realized near the location. The research site itself has remained undeveloped and is currently used as a quay with walkway. As far as it is known, no soil–threatening activities have taken place since the soil survey in 2008 (described further).

The total square of the site location is 600 m² (100 x 6 meters), of which 0 m² were built–up. It is unpaved, but has a gravel paved walkway (Figure 2.1).

At the research location, the following soil investigations have taken place (source: joint environmental protection agency of the province of South Holland and 15 municipalities):

- exploratory soil investigation (DCMR, 1 January 1981) – [1]**;
- further soil investigation (DCMR, 1 January 1985) – [2];
- exploratory soil investigation (SGS ECOCARE, January 1, 1989) – [3];
- further soil investigation (SGS ECOCARE, 1 January 1989) – [4];
- NAVOS location reporting (Municipal Works, 23 March 2004) – [5];
- exploratory soil investigation of the former landfill nearby (November 3, 2008) – [6];
- preliminary study (LIEVENSE CSO, 7 March 2017) – [7]

The composition of the selected (mixed) samples and their additional analyzes for the breakdown into spot samples, determination of whether and to what extent soil contamination is present (assessment values are included in the Soil Remediation Circular 2013 – analysis results have been tested against the assessment values from this circular) are shown in the table 2.1 below.

** The references in brackets refer to the reference list at the top of section 2.2.

Table 2.1

Previous report of samples selection and exceedances of threshold values (Circular Soil Remediation Act)

Sample	Sample trajectory	Boring number	Degree of contamination		
	(m – mv–above surface)		> AW	> T	> I
MMBG1	0,00 – 0,60	01 (0,00 – 0,50) 02 (0,25 – 0,60)	Cobalt, Nickel, Zinc, Cadmium, Lead	–	–
MMBG2	0,00 – 0,50	03, 04, 05, 06, 08	Cobalt, Nickel, Mercury	Zinc	Copper, Lead
MMBG3	0,00 – 0,50	07 (0,00 – 0,50) 09 (0,00 – 0,50)	Cobalt, Nickel, Copper, Zinc, Mercury, Lead, PAH 10 VROM	–	–
MMOG4	0,90 – 1,80	02 (0,90 – 1,40) 04 (1,35 – 1,80)	Cobalt, Nickel, Zinc, Mercury, PAH 10 VROM	Lead	Copper
MMOG5	1,10 – 1,95	06 (1,10 – 1,60) 08 (1,50 – 1,95)	Copper, Mercury	Lead	–

02	0,90 – 1,40	02 (0,90 – 1,40)	Cadmium, Mercury, Cobalt, Molybdenum	Nickel	Copper, Lead, Zinc
03	0,00 – 0,50	03 (0,00 – 0,50)	Mercury, Cobalt, Molybdenum	Nickel	pper, Lead, Zinc
04	0,00 – 0,50	04 (0,00 – 0,50)	Copper, Mercury, Molybdenum, Lead, Zinc	–	–
04	1,35 – 1,80	04 (1,35 – 1,80)	Copper, Mercury, Lead	–	–
05	0,00 – 0,50	05 (0,00 – 0,50)	Cobalt, Copper, Mercury, Nickel, Molybdenum, Zinc	Lead	–
06	0,00 – 0,50	06 (0,00 – 0,50)	Copper, Mercury, Lead	–	–
06	1,10 – 1,60	06 (1,10 – 1,60)	Mercury, Zinc	–	Copper, Lead
08	0,00 – 0,50	08 (0,00 – 0,50)	–	–	–
08	1,50 – 1,95	08 (0,90 – 1,40)	Mercury, Lead	–	–
Average degree of contamination with heavy metals:			Cobalt, Mercury, Zinc *, Molybdenum, Nickel,	–	Copper, Lead

> AW: exceeding the background value, (light increased);
> T: exceeding the intermediate value, (moderate increased);
> I: exceeding the intervention value, (strong increased);
–: no exceedance;
*: content for zinc is almost equal to the intermediate value.

Also lead (II) resistant strains were predicted as *Bacillus Shigella* or, *Salmonella, Klebsiella* or *Eenterobacter* and *Enteroccocus* for isolated contaminated soil samples of SUNDAR Industrial Estate (Saleem F. et al., 2015). Profound review of the researches for lead resistance in microorganisms was made by Jarosławiecka A. et al., 2014 as well.

2.2. Methodology

Due to the analysis's time sensitivity, all preliminary arrangements (such as laboratory agreement) and further stages plans had to be done, prior the sampling. The list of laboratories, able to cooperate with, is presented in the annex 5.

The fieldwork for environmental hygienic soil research (from acceptance of the assignment for the fieldwork up to and including the transfer of the field data, fieldwork notes and samples to SWECO) was carried out under process certificate BRL SIKB 2000 (Field work for environmental soil research, version 5, December 2013 and the protocols 2001 'Placing hand drillings, making characterization of the soil, taking soil samples'.

Following receiving laboratory responsibilities and steps:

- DNA isolation from starting material (the "QIAGEN DNEASY POWERBIOFILM" kit was used);
- quality control for each sample;
- preparation of the 16S amplicon sequencing libraries;
- quality control of the sequencing libraries;
- sequencing on "Illumina MISEQ" sequencer with the "MISEQ Reagent Kit v2" – 500cycle;
- read length is 250 cycles one way and 250 cycles the other way of a 457 base pairs DNA amplicon.
- delivery of raw data (FASTQ files), used for the downstream analysis.

For the downstream analysis the MOTHUR pipeline was used. This pipeline assembles forward and reverse runs, remove unreliable and chimeric sequences, perform alignments, define operational taxonomic units (at 97% sequence similarity), used to classify groups of closely related individuals and then perform taxonomic identifications of the OTU's (Schloss P. et al., 2009). The output (after the sequence part and the MOTHUR pipeline) is between 50.000–100.000 sequences per sample.

2.3. Fieldwork

The visual inspection and soil sampling were carried out on 24th of July 2018, between 14:00 and 16:00. It was sunny during the field research, 30°C on average according to "ACCUWEATHER". There was a wind of 14 km/hour south–eastern direction
– "TIMEANDDATE" online service. Based on dispersion risks, the weather conditions were appropriate for soil sampling and its visual inspection. Table 2.2 contains the boreholes profiles description (as was already mentioned, top soil layer – 0,5m.), complementing the previous SWECO's site report.

The field work consisted of the following activities:

• performing a visual terrain inspection. Partly on the basis of this, the location of the previous drillings has been detected according to GPS coordinates;

- performing a total of five hand drillings with Edelman auger per 3 samples each;
- taking samples of the soil material during the drillings..

Gloves and aseptic liquid (95% ethanol) were used to rinse the equipment before and after each sample. Specialized sampling glass jars were provided by the lab.

Delivery was made on the next day within 18 hours after sampling with storage performed in an aforementioned insulated fridge container with ice packs.

Table 2.2

Boreholes profile description

Borehole position number	Description
3	Clay, strong sandy, moderate humus content, strong sand, strong cinders, moderate slag, moderately debris, no oil–water reaction, dark brownish–gray, 30% soil moisture, 45% admixture.
5	Clay, moderately sandy, moderate humus content, weakly debris, remnants of pottery, moderately root–bearing, remains of gravel, no oil–water reaction, dark brown, 20% soil moisture, 3% admixture.
7	Clay, strong sandy, moderate humus content, strong sand, heavily debris, chunks of wood, moderate slag, no oil–water reaction, light gray–brown, 20% soil moisture, 25% admixture.
8	Clay, weak sandy, weak humus content, moderately sandy, residual debris, remnants of plants, remains of plastic, no oil–water reaction, neutral brown, 20% soil moisture, 2% admixture.
9	Clay, moderately sandy, strong humus content, heavily debris, residues of asbestos and roof plates, moderately sandy, chunks of clay, no oil–water reaction, dark brown, 25% soil moisture, 33% admixture.

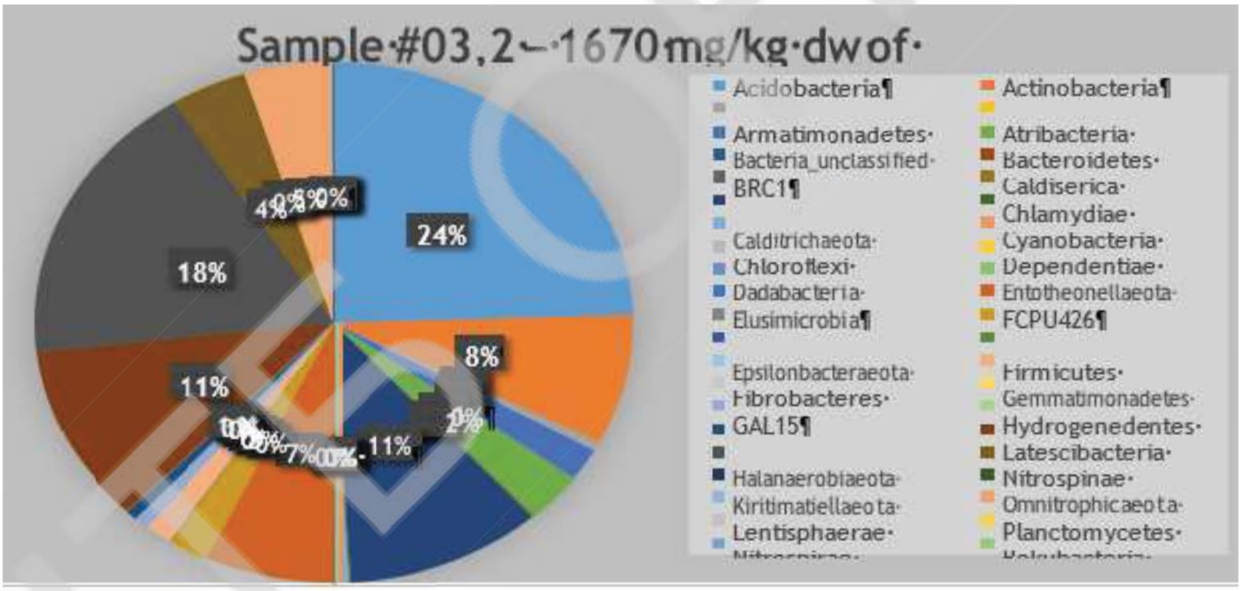
Samples were homogenized, by thoroughly shaking the bag in the jars before shipping. All the samples were stored in the fridge with ice packs, jars were additionally wrapped in the cupboard box.

Unit 3
Study results

3.1. Results and their interpretation

Generally, there were obtained 15 samples, among them 5 were sent to the appropriate laboratory – microbiological laboratory of KWR Water–cycle Research Institute, Utrecht, Netherlands. Selection of samples was based on the height maps of Netherlands, visual soil inspection and boreholes profile (table 2.2).

For the DNA extraction the lab was using "QIAGEN DNEASY POWERBIOFILM" kit. "Illumina MISEQ" sequencer with the "MISEQ Reagent Kit v2" (500cycle) were used as a sequencer and its kit respectively. The output data was outsourced in the "FASTQ" files which are used for the downstream analysis. According to the NGS results given, *Acidobacteria* (up to 26% – max number of reads in one particular replicate), *Proteobacteria* (up to 20%), *Actinobacteria* (up to 15%) and *Firmicutes* (up to 12%) bacteria phyla are dominant in the sampled soil. The most frequently met bacteria genus in the sequencing reads are unclassified *Bacillales, Rhizobiales, Rokubacteriales* and *Candidatus Xiphinematobacter* and others.



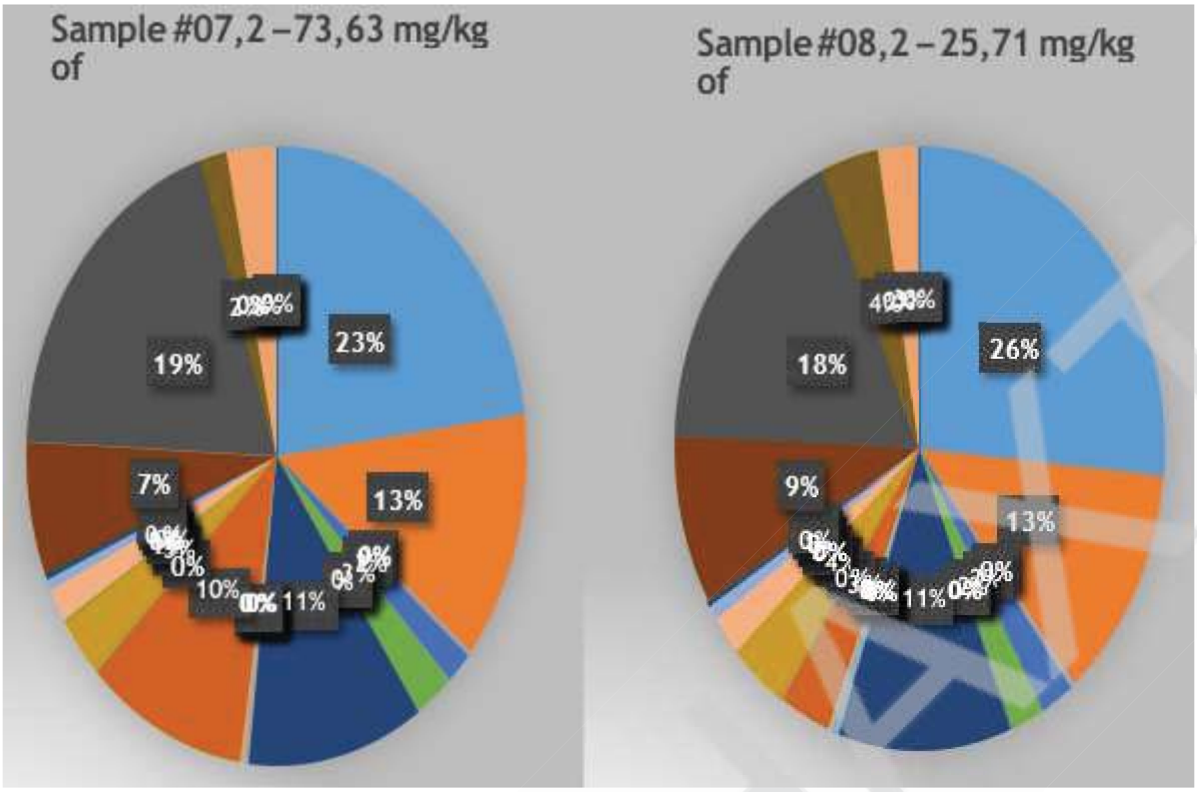


Fig. 3.2. Correlation of soil pollution concentrations and phyla type distribution in the specified samples

One of the reasons to the fact, that the most abundant phylum found – is *Acidobacteria* is that this phylum was also observed to be found of higher quantity in metal–contaminated soils and sediments according to Thrash J. and Coates J., 2015. However, compared averaged replicate samples reads (sample #03,2, #07,2 and #08,2) don't show the same correlation as Thrash J. and Coates J., 2015, observed (fig. 3.4).

3.2. Discussion and prospects

Comparing the results of NGS analysis and the review made in the annex 1 – a few similar genera were found, such as: *Xanthobacteraceae*, *Sphingomonas*, *Actinobacteria*, *Corynebacteriales*, *Bacillus*, but due to the same small number of reads per sample it is almost impossible to identify exact specie, which was found in the soil to match them with one's different scientists found, mentioned in the section of the literature review. Since one genus can include different species, talking about some features of one specie and traversing them to another specie in the same frame of one genus is nonobjective, only continuing further studies and lab analysis with higher quality results output will suffice.

Azarbad H. et al., 2016 and Mikiya H., 1992 mention: "However the application of advanced molecular techniques theoretically warrants more comprehensive and better assessment of microbial communities exposed to pollution factors, still a number of important issues and remarks remain unresolved, among them are: specific soil type and characteristics, metal mixture, type of pollution velocity (rapid/gradual), microbial adaptation rate towards pollution, on site fertilizer application and cropping, and each of these and other factors and its' combinations have to be thoroughly considered.

Also several studies reported no correlation between microbial diversity and metals due to the low concentration or relatively low toxicity of tested metals. (Gołębiewski M. et al., 2014; Niklińska M. et al., 2006; Mikiya H., 1992). This might be one of the reasons for having such an efficiency of the laboratory results delivered in current study.

As for the prospects for this study – more accurate indicators can be used as an assessment tool, due to the sensitivity of microorganisms to low concentrations of the pollutant. Accumulating the similar data for other types of pollution and pollution concentration ranges, etc. will provide sufficient database and solid system of references.

Conclusion

This progress report revealed physicochemical site description with the microbial content of the sampled soil at particular site location. The fieldwork for environmental hygienic soil research (from acceptance of the assignment for the fieldwork up to and including the transfer of the field data, fieldwork notes and samples to SWECO) was carried out under process certificate BRL SIKB 2000.

According to the reports' objective – next generation sequencing analysis was performed for lead and cooper polluted soil. Colored plot in annex 6 represents the community composition of bacteria phylum present in the current soil samples. Figure 3.4 depicts diagrams for correlation of soil pollution concentrations and phyla type distribution in the specified samples.

Acidobacteria (up to 26%), *Proteobacteria* (up to 20%), *Actinobacteria* (up to 15%) and *Firmicutes* (up to 12%) bacteria phyla were shown to be dominant in the sampled soil. The most frequently met bacteria genus in the sequencing reads are unclassified *Bacillales*, *Rhizobiales*, *Rokubacteriales* and *Candidatus Xiphinematobacter* and others. But one of the reasons, that the most abundant phylum in this ranking is *Acidobacteria* – is that this phylum was also observed to be found of higher quantity in metal–contaminated soils and sediments according to Thrash J. and Coates J., 2015. Thus, it might be used as an indicator tool as well, however, unfortunately in current situation the same correlation has not been found.

General structure and the approach way of the survey could and should also be implemented for other types of pollutants, their mixture, other soils, their vegetative conditions, etc. The further future works needed and have to be carried out. Next steps for the project: polymerase chain reaction analysis of the samples with the pollution exposure, anti–metal resistance patterns testing, binding the correlation between the concentration of the pollutant and its impact on the microbial population.

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