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MICROBIAL SCREENING AT THE BUKOV URF AND THE ROŽNÁ MINE - FINAL REPORT OF THE PROJECT

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et al.

Technical University of Liberec

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SÚRAO

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Final report of the project

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Contents

List of figures:

List of tables:

List of attachments:

Attachment 1: List of samples “Priloha1_seznam_vzorku.xlsx”

Attachment 2: List of dominant OTU “Priloha2_dominantníOTU.xlsx”

List of abbreviations:

APS	adenosine 5′-phosphosulphate
CFU	colony forming units
DNA	deoxyribonucleic acid
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
DGR	deep geological repository
IRB	iron-reducing bacteria
NGS	next generation sequencing
NIR	nitrite reductase
NRB	nitrate reducing bacteria
OTU	operational taxonomic unit
PCA	principal component analysis)
PTFE	polytetrafluorethylene (Teflon)
qPCR	quantitative polymerase chain reaction
SOB	sulphur-oxidising bacteria
SRB	sulphate reducing bacteria
TAE	terminal electron acceptor
TPC	technically pure culture
TTC	triphenyl tetrazolium chloride
TUL	Technical University of Liberec
URF	underground research facility

Abstrakt

Cílem této zprávy je shrnout výsledky mikrobiologického průzkumu v oblasti PVP Bukov a dolu Rožná. Analyzovány byly vzorky vod a mikrobiálních nárůstů z oblasti 12. a 24. patra. Pro stanovení mikrobiální diverzity byly použity především molekulárně biologické metody (konkrétně amplikonová sekvenace oblasti 16S rRNA a qPCR) a jako doplněk pro část vzorků i kultivační postupy. Celkem bylo odebráno 122 vzorků pro molekulárně-biologické postupy a 7 vzorků pro kultivační analýzy. Důraz byl kladen na zachycení funkční diverzity mikrobiálních společenstev.

Výsledky mikrobiologických analýz prokázaly, že mikrobiologická diverzita ve studovaném území je silně ovlivněná aerobním prostředím důlních chodeb. Obligátně anaerobní mikroorganismy byly zjištěny pouze v omezeném množství. Mezi nejhojněji zastoupené funkční skupiny patří mikroorganismy schopné oxidovat organické látky, sloučeniny železa a síry. Až na vzácné výjimky je mikrobiální diverzita v jednotlivých vrtech poměrně stabilní v čase. Výsledky kultivačních analýz v zásadě odpovídají výsledkům molekulárně-biologických analýz.

Pro spolehlivější výsledky mikrobiologických analýz byl vyvinut vzorkovací systém z uzavřených vrtů. Hlavní součástí tohoto systému je pasivní vzorkovač sloužící k zachycení mikrobiální biomasy v podzemní vodě. Tento vzorkovací systém minimalizuje vliv aerobního prostředí důlních chodeb a eliminuje tak vliv antropogenních zásahů na lokalitě. Je vhodný pro dlouhodobý monitoring mikrobiologických změn. Na vrtech, kde byl umístěn vzorkovací systém, proběhla i hydrochemická in-situ měření.

Na základě výsledků tohoto mikrobiologického průzkumu byla navržena doporučení pro budoucí aktivity zahrnující mikrobiologické analýzy.

Klíčová slova

Mikroorganismy, mikrobiální nárůsty, pasivní vzorkovač, podzemní voda, PVP Bukov

Abstract

The aim of this report is to summarise the results of the microbiological screening in the area of the Bukov URF and the Rožná mine. The groundwater samples as well as the samples of microbial mats collected on the 12th and 24th level were analysed. Molecular-biological methods (specifically, amplicon sequencing of 16S rRNA and qPCR) accompanied by cultivation techniques (for part of the samples) were used in order to determine the microbial diversity. Altogether, 122 samples and seven samples were collected for the molecular-biological and cultivation analyses, respectively. The emphasis was given to the evaluation of the functional diversity of the microbial communities.

The results of the microbiological analysis proved that the microbial diversity at the study area is strongly influenced by the aerobic environment of the mine workings. Obligate anaerobic microorganisms were detected only in limited abundancies. Microorganisms capable of oxidation of organic, iron and sulphur compounds were found to be the most common functional groups. The microbial diversity in the studied boreholes is relatively stable over time (with rare exceptions). The results of cultivation analysis basically corresponded to the results of the molecular-biological analysis.

The sampling system for the water samples from the closed boreholes has been constructed in order to obtain more reliable results of the microbiological analysis. A passive sampler for capturing microbial biomass present in the groundwater represents the principal component of this system. This sampling system minimises the effect of the aerobic environment of the mine workings and eliminates the effect of the anthropogenic activities at the study site. It is suitable for the long-term monitoring of microbiological changes. The boreholes, where the passive samplers were installed, were monitored for hydrochemical parameters.

Recommendations for the future activities that include microbiological analysis have been proposed, based on the results of this microbiological investigation.

Keywords

Microorganisms, microbial mats, passive sampler, groundwater, Bukov URF

1 Introduction

Stage 2 of this project follows on the preparatory work carried out in the framework of Stage 1 (Steinová et al. 2018), in which reconnaissance and initial sampling of accessible areas of the Bukov URF and the Rožná mine were carried out and a passive sampler used to capture microbial biomass in groundwater was created and installed.

The submitted final report summarises all of the results of the microbiological screening at the Bukov URF and the Rožná mine. The main objective of this project (according to the task description) was to describe the microbial colonisation of the rock environment of the Bukov URF and the Rožná mine with the expected use of the obtained data for several follow-up experiments focused on the study of the resistance of engineering barriers. Another aim was to distinguish between natural and anthropogenically affected communities.

During the course of Stage 2, all of the available groundwater sources suitable for regular monitoring at the Bukov URF were repeatedly sampled. Other parts of the mine were repeatedly sampled from boreholes S-22, S-23, S-24, S-25 and S-26 (all on the 12th level). In the case of these boreholes, sampling was performed using a passive sampler.

All of the collected samples were subjected to molecular-biological microbiological analysis (in particular amplicon sequencing of the 16S rRNA region and quantitative PCR), and some of the samples were also analysed using culture techniques. The main output of the analysis was the determination of the functional profile of the microbial communities occurring in the study area. Furthermore, the submitted report will serve as a basis for the proposal of other activities in which the microbiological conditions at the Bukov URF and the Rožná mine will be evaluated.

2 Sampling of groundwater and microbial growth

Sampling at the Rožná mine and the Bukov URF took place between May 2018 and January 2019. Samples suitable for collection and regular monitoring were selected based on prior reconnaissance.

All of the samples were taken to capture the widest possible range of microorganisms growing in the deep mine and, if possible, to capture microbial communities growing in an environment without anthropogenic interference (similar to the conditions of a deep geological repository).

2.1 Sampling methodology

The sampling methodology for the microbial DNA analysis in the Rožná mine can be divided into two groups according to the nature of the sampled material.

The first group of samples were made up of groundwater samples. Groundwater was obtained either from the outflow of boreholes in the corridors of the mine or from seepages from natural geological disturbances. The water was collected using sterile disposable gloves and sterile collection containers and filters. If the amount of outflowing water was sufficient, the water was taken directly from the site of the outflow with a 50 ml sterile disposable Omnifix syringe (BBraun, Switzerland). If the nature of the outflowing water was unsuitable for this procedure, the water was first collected into a 0.5 l sterile bottle and then sucked through the syringe and filtered. In cases where a tipping shuttle flow meter (most of the outflows at the Bukov URF) was installed at the collection point and there was not enough space upstream to collect the water, the water was collected using a 50 ml syringe directly from the shuttle. If a pipe with a tap was installed in the area of the borehole, then the water in the pipe was drained for at least three minutes and then the water was collected into a sterile PET bottle. The collected water (approximately 500-1,000 ml based on the flow of the individual boreholes) was filtered on-site (or approximately 4h later) using sterile Sterivex filters (Millipore, Germany) (Fig. 1). Sterivex filters capture the microorganism cells present in the water and allow for easy handling during transport and subsequent DNA isolation. They also reduce the risk of any possible contamination by unwanted microorganisms, which is an important advantage when collecting samples of water with a low concentration of microorganisms (typically groundwater). The Sterivex filters with the captured microorganisms were stored in sterile bags, frozen prior to transport to TUL, and then transferred to TUL in cool boxes with cooling blocks to a freezer set at a temperature of -80°C, where they were stored for subsequent DNA isolation. Another type of sampling was the sampling of groundwater using a passive sampler installed directly in specific boreholes. This type of sampling is described in the next chapter (chapter 3).

The second type of sampling was solid phase smears and scrapings. This type of sampling relates to areas with obvious microbial activity in the mine corridors, for which fine microbial mats on outflows from boreholes, expansive, differently-coloured streamers with mineral phase on fractures or from the mouths of boreholes, gelatinous slimy formations sometimes leaking as "hard waterfalls" and reaching a volume of up to hundreds of millilitres, soft organic cauliflower-like formations (usually strikingly orange) blooming in several places on the mine floor, where water was retained, can be considered. These solid phase samples were collected into sterile 50 ml or 15 ml tubes (so-called falcons, Fig. 3) using disposable sterile gloves or if necessary using disposable sterile spatulas (Fig. 4) or a scalpel, which was sterilised over a flame before each collection. The solid phase samples were also frozen prior to transport to TUL and then transferred to TUL in cool boxes with cooling blocks to a freezer set at a temperature of -80°C, where they were stored for subsequent DNA isolation. The sampling equipment used for sampling are shown in Fig. 1.

A more detailed description of the sampling and characterisation of the sampling sites are provided in the table of samples (see the table of samples).

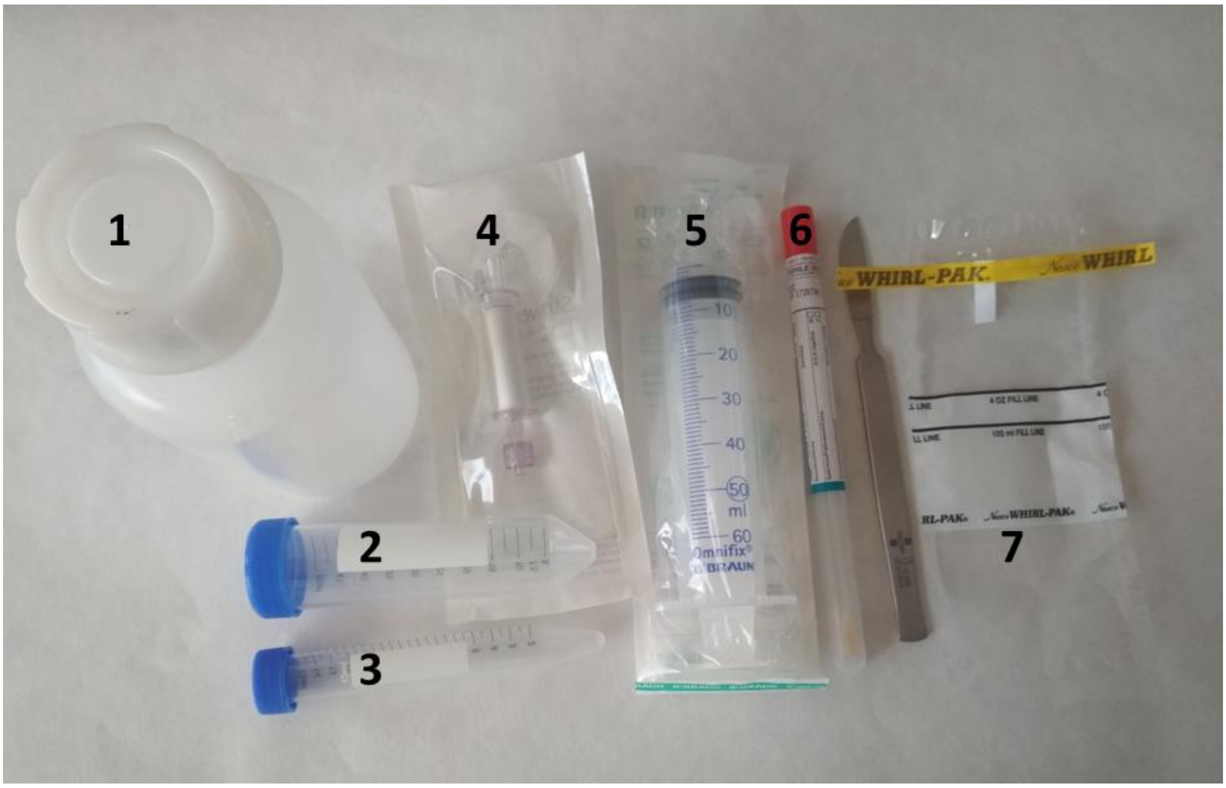

Fig. 1 Sampling equipment used: 1 - sterile 500 ml bottle, 2 - sterile 50 ml tube, 3 - sterile 15 ml tube, 4 - Sterivex filter, 5 - 50 ml syringe, 6 - sterile disposable spatula, 7 - sterile bag for the storage and transport of the Sterivex filters

Fig. 2 Filtering water using a Sterivex filter

Fig. 3 Sampling of microbial mats into a sterile tube (so-called falcon)

Fig. 4 Sampling of microbial growths using a sterile disposable spatula

2.2 Results

Altogether, 122 samples of groundwater and microbial mats were collected from the Rožná mine. A total of 98 samples were collected on the 12th level and 24 samples on the 24th level. On the 24th level, outflows from boreholes and fractures had a low yield for water sampling, and therefore samples of microbial mats (microbial mats - 22 samples, water – two samples) prevailed. On the contrary, on the 12th level, there was a sufficient outflow of water - 64 samples were collected of water filtered using a Sterivex filter, five samples of water were collected using a passive sampler and 29 samples of microbial mats were collected. A list of the samples is given in Attachment 1.

3 Sampling system for microbiological analysis, its installation and sampling

The sampling system is based on a passive sampler that was designed during Stage 1 (Steinová et al. 2018). The system was improved in several ways. The current version is fully sterilisable, easy to install and easy to modify.

Similar equipment was installed on boreholes with greater groundwater outflows and allowed the microorganisms to be retained in this water. They are used to capture the spectrum of native species of microorganisms present in anaerobic groundwater (which will crucial for the future long-term development of a deep geological repository). Furthermore, they allow to capture a greater volume of biomass, minimise the impact of the aerobic environment of the tunnel/built repository and eliminate the impact of the current anthropogenic interventions at the site.

3.1 Description of the sampling system

A sampling system was assembled for sampling microbial communities (Fig. 5). During the assembly, emphasis was placed on the possibility to sterilise the individual parts of the passive sampler, i.e. inlet hoses and filter holder, especially with a view to minimising possible sample contamination. At the same time, it was necessary to solve the filtration without the inlet of air (under anaerobic conditions) and minimise contact between the groundwater and O_2 air in the boreholes. Due to the low pressure in the monitoring boreholes, which does not allow sufficiently rapid spontaneous filtration, the passive sampler was supplemented with further components, which accelerate the groundwater filtration. The sampling was based on the principle of filtration under reduced pressure.

A stainless-steel mechanical obturator with a length of 1 m was used for sealing the borehole with the sealing part having being 150 mm long and 74 mm in diameter. A quick coupling was installed on the mouth of the obturator to connect a PTFE tube with a diameter of 10 mm to which a passive sampler was attached (Fig. 7).

A tube with a 10 mm diameter was chosen with respect to the outflow measured in the boreholes to avoid "throttling" the flow. A 6-mm diameter PTFE tube was used for boreholes with lower flow. A durapore filter with a diameter of 47 mm and a porosity of 0.22 µm (Merck, Germany) (Fig. 8) installed in a flow-through stainless steel filter holder (Sartorius, Germany) was used to capture microbiological communities, allowing for sterilisation and filtration at pressures of up to 20 bar. Collecting the filtrate and creating a stable vacuum were accomplished using a transition vessel (Fisher, Germany, 2 litres). The vacuum was created using a LABOPORT® membrane vacuum pump (flow rate 10 l/min, P_{max} = 8 mbar (abs)). Due to the limited connection to the 220 V electricity mains in the underground space, the vacuum pump was powered by a 12V/20 Ah battery and a 12/220 V, 550 W voltage converter. Vacuum cylinders (13 litre containers) were subsequently used to collect samples from multiple boreholes at the same time (Fig. 6). The whole system was installed with quick couplings (Ni-Cr), which can be thermally sterilised (up to 200°C) and allow for variability and easy modification of the whole system.

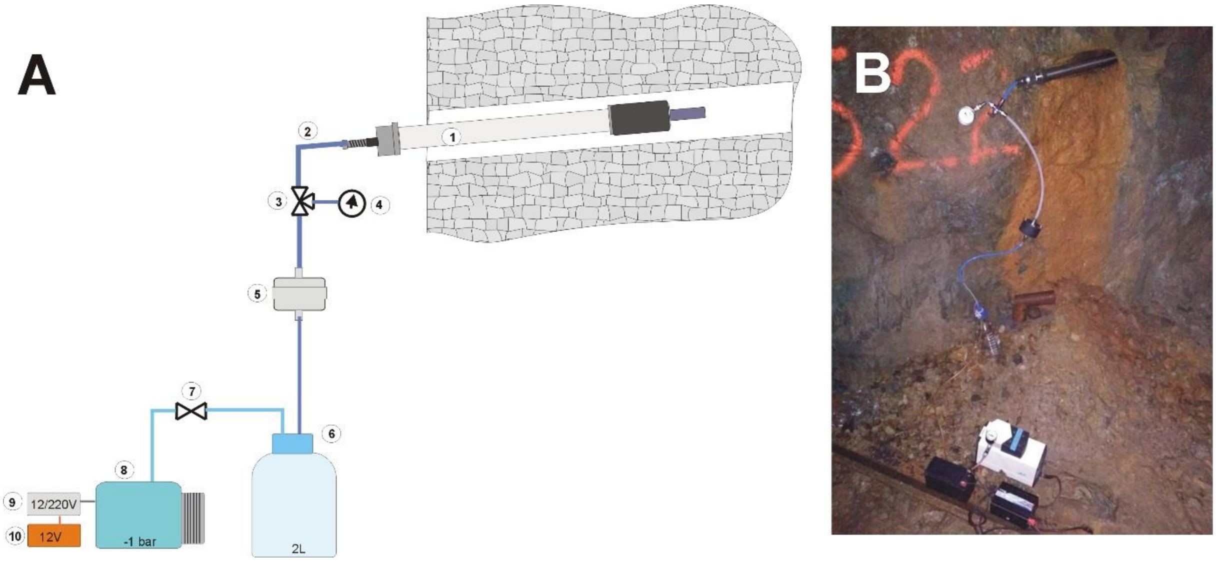

Fig. 5 Representation of the complete system for the collection of microbiological samples from the groundwater. A: Description of the components used: 1) Stainless-steel mechanical obturator, 2) PTFE tube (Ø 10 mm), 3) Three-way valve, 4) Pressure gauge/pressure sensor, 5) Filter holder (Sartorius) 6) Fisher transition vessel (2 litre volume)) 7) Ball valve 8) Membrane vacuum pump 9) 12/220 V voltage convertor 10) 12V battery. B: Image of the sampling system during the collection of a sample from borehole S-22

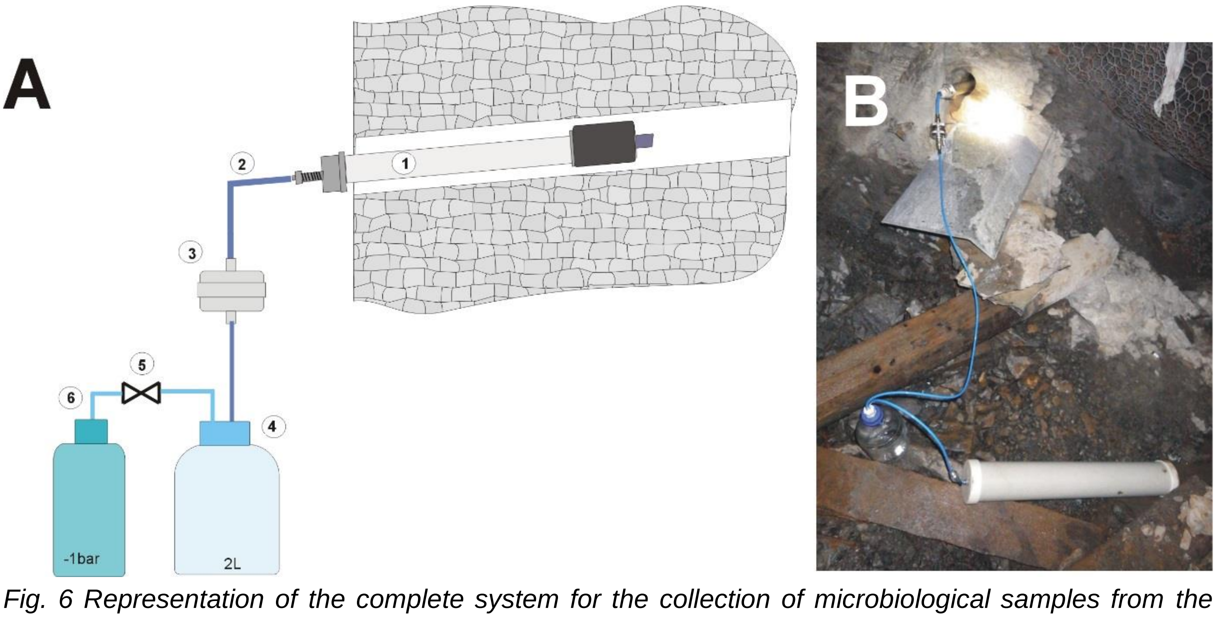

Fig. 6 Representation of the complete system for the collection of microbiological samples from the groundwater - alternative 2 using a vacuum cylinder. A: Description of the components used: 1) Stainless-steel mechanical obturator, 2) PTFE tube (Ø 10 mm), 3) Filter holder (Sartorius), 4) Fisher transition vessel (volume 2 litres), 5) Ball valve, 6) Pressure vessel with vacuum. B: Image of the sampling system during the collection of a sample from borehole S-26.

Fig. 7 Connection of a sample with a PTFE tube

Fig. 8 Durapore filters for capturing biomass

3.2 Installation of the sampling system and sampling for microbiological analysis

The boreholes were measured before the packers were installed in the individual boreholes. After the test installation of the stainless-steel packers, the tap was closed on the borehole mouth and after the conditions were stabilised, the flow rates from the borehole were measured (Tab. 1) as well as the approximate pressure values. The final sampling system was designed based on the results of the measurements. The pressure values were not possible to measure or they were very low (boreholes S23, S25), so the passive sampler was supplemented with components for performing the filtration under reduced pressure. For passive filtration, a pressure of at least 0.8 bar is required relative to the filter resistance.

Tab. 1 Flow values in the monitoring boreholes

Borehole	Date	Flow (l/min)	Note
S-23	15/11/2018	0.777	Before installing the packer
S-23	15/11/2018	1.429	After installing the packer
S-26	15/11/2018	0.577	Before installing the packer
S-26	15/11/2018	1.872	After installing the packer
S-25	29/11/2018	0.641	Before installing the packer
S-22	29/11/2018	Low (not measurable)	Before installing the packer
S-24	29/11/2018	Low (not measurable)	Before installing the packer
S-24	9/1/2019	1.205	After stabilising (5 weeks)
S-22	9/1/2019	0.031	After stabilising (5 weeks)
S-25	9/1/2019	0.603	After stabilising (5 weeks)
S-26	9/1/2019	1.695	After stabilising (7 weeks)
S-23	9/1/2019	1.304	After stabilising (7 weeks)

The packers were installed in the monitoring boreholes in advance. The packers were installed in boreholes S-23 and S-26 on 15 November 2018, and in boreholes S-22, S-24 and S25 on 29 November 2018. The boreholes were left sealed with natural discharge (Tab. 1) for more than five weeks until natural stabilisation of hydrochemical and microbiological conditions was obtained. In the case of borehole S-22, the groundwater level was led approximately 1 m above the mouth of the borehole to ensure thorough venting and ensuring, where possible, anaerobic conditions on 29 November 2018; in the other boreholes this occurred on 9 January 2019.

Fig. 9 Sealing of borehole S-22 using a mechanical obturator with the discharge led approx. 1 m above the mouth of the borehole (stabilisation of conditions)

A durapore filter with a diameter of 47 mm and a porosity of 0.22 µm (Merck, Germany) was installed in advance in a sterilised filter holder (Sartorius, Germany) under sterile conditions. The filter holder and the PTFE tube were sterilised by autoclaving. After being transported to the underground space, the sterilised PTFE tube was first connected to a packer and flushed with groundwater from the borehole. Subsequently, a passive sampler was connected to a vacuum filtration system. Expect for borehole S-22, it was possible to filter the water to a limited extent with the pressure in the boreholes without the need for a vacuum, but in most cases the filtration was very slow.

After connecting the filter cell, a vacuum was created and the water in the range of 1-3 litres was filtered through the filter. Subsequently, the filter cell with the filter was detached, placed in a sterile PE container and transported in a transport refrigerator to the molecular biology laboratory of TUL to analyse the captured biomass. The filters were removed from the holders in the laboratory under sterile conditions and subsequently frozen until further processing. The collected filtrate can be used for hydrochemical analysis.

3.3 Measurement of hydrochemical parameters

3.3.1 Methodology

The basic hydrochemical parameters (temperature, pH, Eh, O_2; see Fig. 11 - Fig. 15) were measured to monitor the steady-state hydrochemical conditions. After collecting the microbiological samples, a flow cell was connected, which allowed flow measurements to be taken without access of air (Fig. 10). After connection, the cell was thoroughly flushed with groundwater and the measured values were recorded.

Fig. 10 Measurement of the basic hydrochemical parameters (temperature, pH, ORP, O_2) in the flow cell

Measurements were taken using a WTW 3630IDS device with ORP probes: WTW ORP T_900P, pH: WTW SenTix94X_P, conductivity: WTW TetraCon925_P, O2: WTW FDO925_P and IDS WLM wireless data transmission. The electrodes were calibrated prior to the measurements using the following standards NIST WTW pH 4.01, 7.00 and pH 10.01; WTW E-SET Trace 0.01 N KCl. The zero point of the O_2 probe was controlled in a sodium sulphite solution according to DIN EN 25814/ISO 5814. Calibration of the ORP probe was performed using a WTW RH28 standard. The ORP values were converted to a hydrogen electrode (U_{ref} /+214 mV).

3.3.2 Results

Measurements in boreholes S-22, S-23, S-24, S-25 and S-26 were performed in the flow cells after collecting the microbiological samples using passive samplers and the main objective was to gain an idea of the Eh conditions in the boreholes. From the records of the values, it is possible to observe a gradual stabilisation of the values during the measurement. Although steady-state values were not reached, trends between the boreholes could be observed. After approximately 15 minutes, a decrease in Eh values can be observed. The lowest values were measured in borehole S-25, where the Eh values were around - 100 mV and the content of O_2 ranged around the detection limit. In the case of boreholes S-24 and S2-6, a decrease in Eh values to around -40 mV and -60 mV was observed, respectively. High Eh values were measured in borehole S-22 (approximately 200 mV) and the O_2 content was up to 3 mg/L. A unusual course was observed in borehole S-23, which showed a gradual increase of Eh to values around 350 mV and an O_2 concentration of 7.8 mg/l (Fig. 11, Fig. 12, Fig. 13, Fig. 14, Fig. 15).

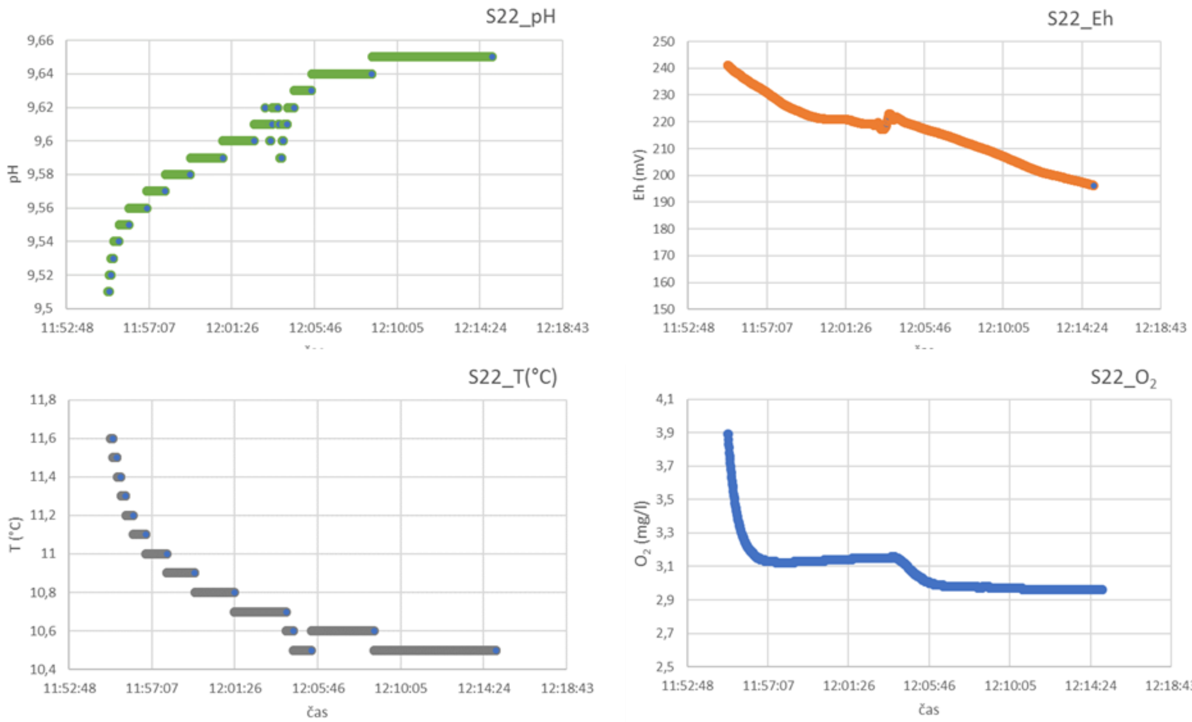

Fig. 11 Record of hydrochemical parameters (pH, Eh, T, O_2) measured in the flow cell (borehole S-22)

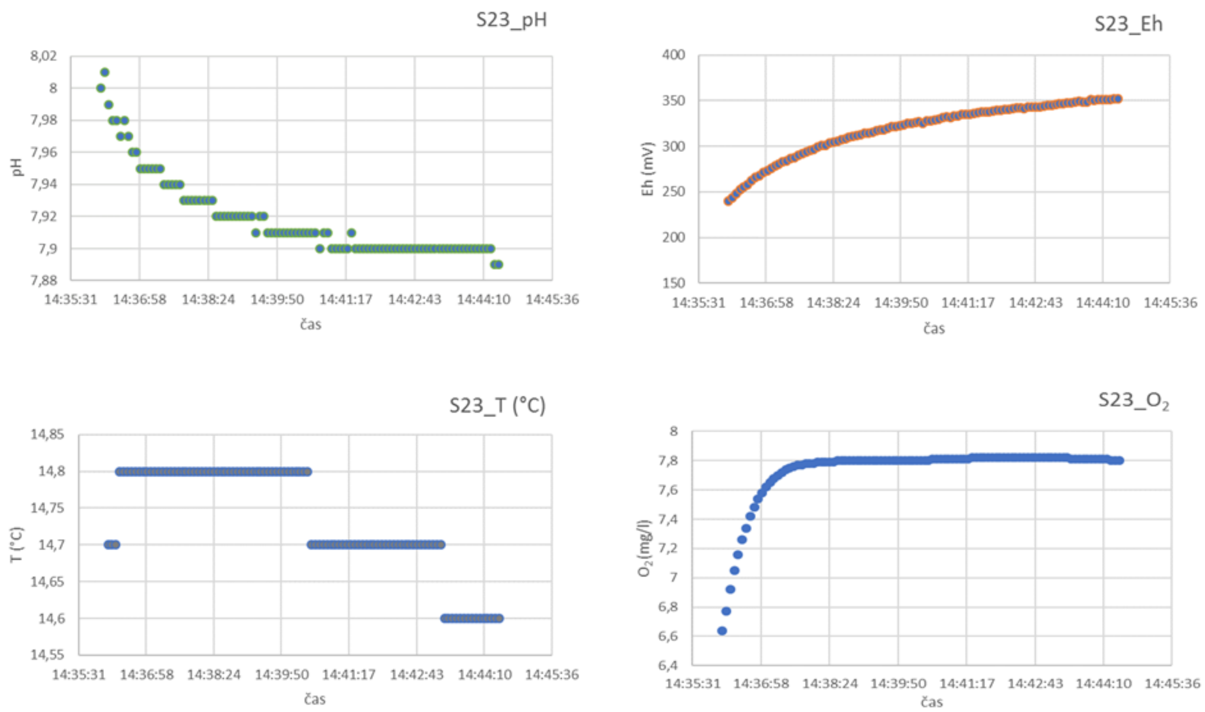

Fig. 12 Record of hydrochemical parameters (pH, Eh, T, O_2) measured in the flow cell (borehole S-23)

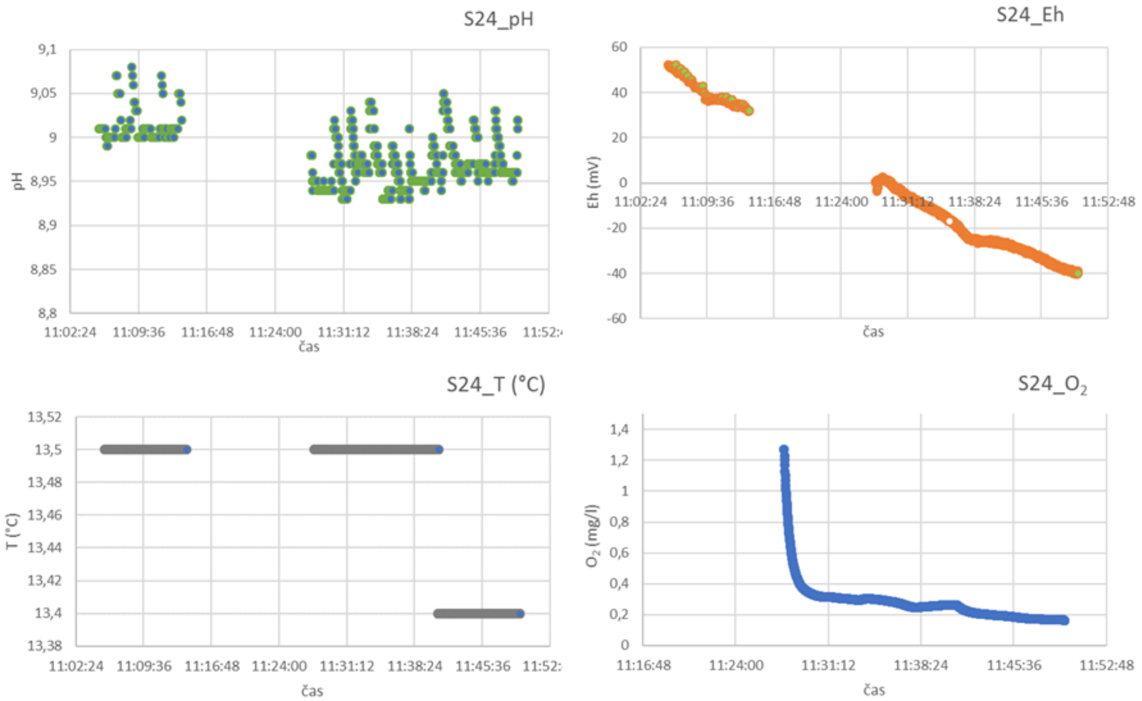

Fig. 13 Record of hydrochemical parameters (pH, Eh, T, O_2) measured in the flow cell (borehole S-24)

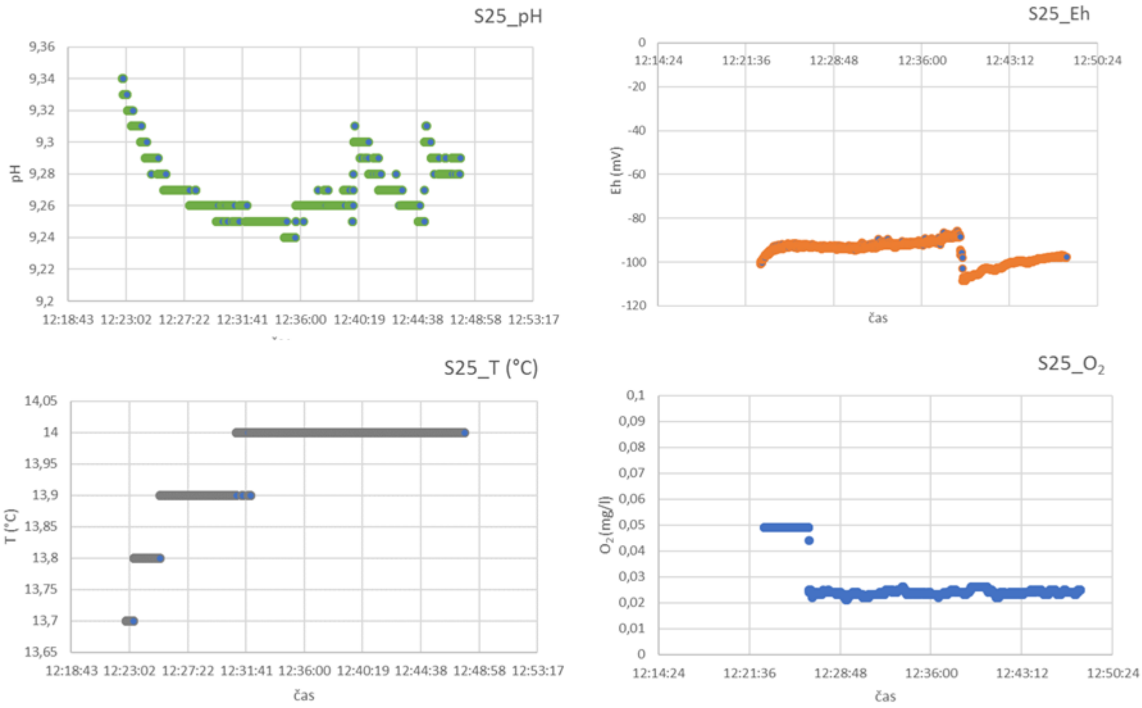

Fig. 14 Record of hydrochemical parameters (pH, Eh, T, O_2) measured in the flow cell (borehole S-25)

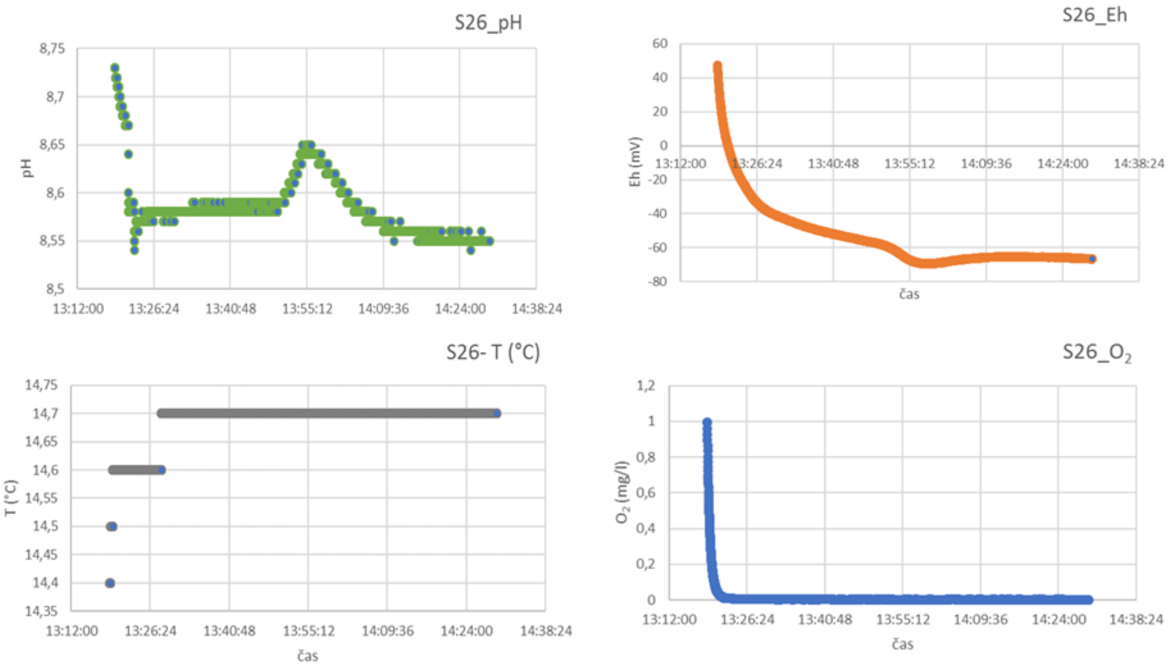

Fig. 15 Record of hydrochemical parameters (pH, Eh, T, O_2) measured in the flow cell (borehole S-26)

4 Molecular biological microbiological analysis

All of the collected samples were subjected to a molecular biological analysis in order to study the diversity of the microorganisms using DNA-based methods. These procedures essentially capture the complete composition of the microbial community present. Some of the samples were also analysed using traditional culture procedures (see Chapter 5) to provide information on the cultivable part of the microbiological spectrum (up to 15 %; Amman et al. 1995) and to compare with the molecular biological approaches.

4.1 Methods used

DNA-based procedures were used to study the microbial diversity in both the groundwater and the microbial samples. The PowerWater DNA isolation kit (MoBio, USA) was used to extract DNA from the groundwater and the microbial samples. DNA extraction was performed according to the kit manufacturer’s protocols. The concentration of DNA obtained was then measured using a Qubit® 2.0 fluorometer (Invitrogen, Life Technologies, USA) according to the manufacturer’s protocols.

New generation sequencing (NGS) methods, i.e. amplicon sequencing of the 16S rRNA genus, were used to study the microbial diversity. The method was described in more detail in the interim report of Černík et al. (2015). The IonTorrent platform (Life Technologies, USA) was used. The V4 variable region was amplified using 530F (Dowd et al. 2008) and 802R (Claesson et al. 2009) primers.

The Seed software version 2.04 (freeware, from the Laboratory of Environmental Microbiology, Institute of Microbiology ASCR; Větrovský and Baldrián 2013) was used for processing the NGS data. This program removed sequences with a quality of less than 25 from the sequence set. All of the sequences shorter than 250 bp (including tags and primers) were subsequently removed. The sequences were categorised into samples based on their tags and then the tags were cut. These sequences were then aligned in the MAFFT program using the FFT-NS-2 method (Katoh et al. 2002) and the primers were removed. After aligning the sequences, those sequences that were not read by the end of the desired segment were manually deleted. Leaving such sequences in the dataset would create artificial diversity of the OTU (operative taxonomic units). OTU were created from the retained sequences using the UPARSE tool in the Seed program, which also eliminates the chimeric sequences. For each individual OTU, the standard similarity limit was 97%. Representative sequences were created for the OTU in the same program, which serves to compare the individual OTU with public databases. The publicly-available GenBank database was used for taxonomic ranking. Sequences that occurred only once in the dataset (so-called singletons) were not taken into account when interpreting the results because they may represent a relatively high probability of sequencing error. Subsequently, the designated taxa were metabolically characterised according to the available literature. In this way, the most abundant microorganisms in the samples were characterised so that at least three of the most abundant taxa were metabolically determined in each sample. The table of individual OTU in the samples was normalised to 10,000 sequences/sample to make the samples comparable to each other. Thus, each number in the microorganism table represents the number of 16S rDNA amplicon sequences if 10,000 were sequenced per sample. If the samples contained more than 50 % of amplicons whose sequences are 100% identical to typical laboratory or anthropogenic contaminants, the sample was excluded from subsequent analysis. If the sample contained less than 50 % of the contaminants, it was left in the analysis and only the contaminating OTU were discarded. A table of frequencies of the most abundant OTU in the individual samples was created (see the microorganisms table) and from the same data an indirect gradient analysis of PCA was performed in the Canoco program (Lepš and Šmilauer, 2000) to show the microbial diversity of the Bukov URF.

Quantitative PCR (qPCR) was used to detect and relevantly quantify specific functional groups of microorganisms (specifically sulphate-reducing bacteria, denitrifying bacteria and total bacterial biomass). Specifically, genes encoding the expression of adenosine 5' phosphosulfate (APS) reductase (for sulphate-reducing bacteria) and nitrite reductase (NIR) for denitrifying bacteria were amplified. Universal 16S rRNA primers were used to evaluate the total bacterial biomass. More details on the primers used are given in Tab. 2. The analysis was performed on a LightCycler ® 480 instrument (Roche Biochemicals, USA).

Tab. 2 List of the qPCR primers used

Primer	Sequence 5'-3'	Specificity	Source
RH1-aps-F	CGCGAAGACCTKATCTTCGAC	SRB: genes encoding adenosine 5' phosphosulfate reductase	Ben-Dov et al. 2007
RH2-aps-R	ATCATGATCTGCCAGCGGCCGGA		Ben-Dov et al. 2007
nirK-876F	ATYGGCGGVCAYGGCGA	NRB: genes encoding nitrite reductase	Henry et al. 2004
nirK-1040R	GCCTCGATCAGRTTRTGGTT		Henry et al. 2004
16SqPCR-F	TCCTACGGGAGGCAGCAGT	All bacteria: universal 16S rRNA primers	Nadkarni et al. 2002
16SqPCR-R	GGACTACCAGGGTATCTAATCCTGTT		Nadkarni et al. 2002

4.2 Results

From the 122 samples, 101 samples were usable for microbial analysis. There were 21 unusable samples, 13 were due to erroneous sequencing and 8 were due to a high proportion of laboratory or anthropogenic contaminating microorganisms. Of the 21 unusable samples, 16 were taken from the groundwater through the Sterivex filter, two were taken from the microbial mat, and three were from the passive sampler. This is probably due to the low levels of DNA obtained, which is typical for microbiologically poor samples of deep groundwater. When amplifying the DNA, the signal from the foreign DNA sample found in the chemicals and kits used (e.g., Salter et al. 2014) or in the surrounding environment becomes drowned out.

From the sequencing of 109 samples (all of the samples except for those with sequencing error), 1,239,682 sequences (without singletons) were scanned into 11,361 OTU. After removing the highly contaminated samples, contaminating the OTU, and after selecting the most abundant OTU, 510,613 sequences within 131 OTU were used for the microbial diversity analysis and metabolic characterisation of microorganisms as listed in the microorganism table (see Attachment 2: List of Dominant OTU).

4.2.1 Characteristics of the metabolic diversity of the microbial communities

When evaluating the metabolic diversity of microbial communities in the study area in relation to the planned DGR, it is necessary to realise that any microorganism activity - aerobic and strictly anaerobic metabolic groups - is potentially dangerous for the integrity of the individual components of the storage containers. Nevertheless, it is possible to establish functional groups that can be expected to have a significant impact on the safety of the DGR. These are mainly microorganisms involved in microbial-induced corrosion (especially sulphate-reducing and sulphur-oxidising bacteria, iron-reducing and iron-oxidising bacteria and nitrate-reducing bacteria) and microorganisms that have the ability to affect the functional properties of bentonite (especially iron-reducing bacteria). Beyond these groups, any microorganisms that consume or produce gases and microorganisms that produce organic compounds are potentially dangerous. It should also be highlighted that, although at present the bacteria of a particular group are present in very small (and possibly undetectable) numbers, under the appropriate conditions (i.e., sufficient donors and terminal electron acceptors are needed, supplemented by an absence of limiting factors), they can rapidly develop and there may an overall change of species and functional spectrum. As mentioned below, all of the potentially dangerous groups of microorganisms have been detected in the study area.

Microorganisms in terms of the electron donors used

- ***Microbial oxidation of iron***

In the groundwater sample from the Rožná Mine, the most common iron oxidising microorganisms were closely related betaproteobacteria *Gallionella sp., Gallionella capsiferriformans* and *Sideroxydans sp.* These microorganisms are neutrophilic gradient microorganisms, i.e. they grow in a narrow range of low oxygen concentrations. At high oxygen concentrations, abiotic iron oxidation occurs rapidly at a neutral pH, and neutrophil ferric bacteria lose their energy source for growth under such conditions. They are often found in groundwater seepages containing iron, in places where these waters meet an oxygenated atmosphere and are not yet fully saturated with oxygen. They can also be part of microbial mats growing in an oxygen-saturated atmosphere - for example, in a ventilated mine corridor where they occupy places where oxygen only gets in to a limited extent (lower layers of growth). In the Rožná Mine, these microorganisms are abundantly present in water flowing out of boreholes S-14, S-20, S-21, and BK-06, BK-06B, BK-07, BK-23, BK-32, BK- 29/33, BK-35, BK-31, BK-38, BR-52 on the 12th level and in water from borehole GR-07 on the 24th level. Furthermore, they are present in microbial mats and streamers growing below several of the seepages (BR-47, BK-06, BR-52, BK-23, BK-06B). These microbial mats or streamers typically have a brown-orange colour due to the precipitation of oxidised forms of iron.

- ***Microbial oxidation of sulphur***

Microorganisms with the ability to oxidise reduced forms of sulphur (e.g., hydrogen sulphide, sulphur, sulphites) are represented in the groundwater of the Rožná Mine by a more diverse group of bacteria than the iron oxidising microorganisms. These are variously related microorganisms from the strains of *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria* and *Epsilonproteobacteria*. The *Alphaproteobacteria* group is represented by *Roseococcus sp.* and *Paracoccus pantrophus*. Both bacteria are an exception in the groundwater under investigation and each only occur in a single place. *Roseococcus sp.* occurs in a massive microbial growth with a gelatinous structure and pink colour. This formation grows at the mouth of borehole 4913 on the 24th level (sample VZ19) and does not have an equivalent in the investigated sites of the Rožná mine. It is known that *Roseococcus sp.* contains pink pigments and is probably the cause of the colouration. In addition to reduced forms of sulphur, it can also use organic substances as an electron source to metabolise, or be phototrophic in light. *Paracoccus pantrophus* was detected in water from the borehole S26, for which a hydrogen sulphide odour is typical. In addition to reduced forms of sulphur, this bacterium has the ability to draw electrons from ammonium ions or organic matter. Respiration is achieved either aerobically or by using nitrates when there is a lack of oxygen. Other sulphur bacteria are generally repeatedly present at various different sites (see the table of microorganisms). These are usually autotrophic or mixotrophic microorganisms *Sideroxydans sp. Thiothrix unzii, Thiothrix fructosivorans, Thiobacillus sajanensis, Thiobacillus sp., Thioalkalivibrio sp., Sulphuricaulis limicola, Sulphuricurvum kujiense, Sulphuricurvum sp., Sulphurisoma sediminicola, and Sulphuritalea sp.* All of these microorganisms are aerobic or microaerophilic (*Sideroxydans* and *Sulphuricurvum*) or facultatively anaerobic (in this case, nitrate respirators) and occur in an oxygen-containing environment. It can be said that microbial oxidation of sulphur is a common microbial metabolism in the waters of the Rožná mine and is typical especially for boreholes S22, S24, S25, S26 and BR-53, which are located on the 12th level outside the Bukov URF. Sulphur oxidising microorganisms are also present in the water standing on the mine floor on the 12th level (VZ23), in an orange microbial mat on BR-49 and in the water from BR-21. In the Bukov URF, the microbial oxidation of sulphur is typical for water from borehole S2 and seepage BK06, and to a lesser extent this ability is also present in microbial communities in the waters from BK06B, BK07, BK29/33, BK35 and in the orange microbial mat from BK23.

It is interesting to look in more detail at the development of the content of sulphur bacteria over time. Boreholes S-24, S-25 and S-26, where repeated sampling of water and biofilms was performed from May to November (or January), can best serve for this purpose. Tables 1-3 show that a different combination of sulphur oxidising microorganisms is present in each of the boreholes. Borehole S-24 was predominantly dominated by various *Thiobacillus* genotypes, borehole S-25 was dominated by two *Sulphuricurvum* genotypes, and borehole S-26 contained both *Thiobacillus* and *Sulphuricurvum*. Each of the boreholes had a specific evolution of the content of sulphur bacteria over time. Initially in the biofilm from borehole S-24, there was only a small amount of sulphur oxidising bacteria; however, in October there was a significant increase, and in November more sulphur oxidising bacteria *Thiothrix unzii* were added. Borehole S-25 was less variable over time. From May to November, *Sulphuricurvum kujiense* dominated both in the water and in the biofilm, while the second genotype of this bacterium was abundant for the whole time, only disappearing from the water in October. In borehole S-26, *Sulphuricurvum* significantly reduced in October, while *thiobacillus* was still abundant. Changes in the content of these bacteria may have been due to their varying ecological alterations. While *Thiobacillus* can only use sulphur compounds as a source of electrons for respiration, *Sulphuricurv* can also oxidise molecular hydrogen and can also tolerate lower oxygen concentrations than *Thiobacillus*. Similarly, the appearance of *Thiothrix unzii* in the biofilm in November 2018 may have been due to the accumulation of organic substances in the biofilm produced by the purely autotrophic bacterium *Thiobacillus*. *Thiothrix*, on the other hand, can use organic substances as a source of electrons and also as a carbon source for the synthesis of its own organic compounds.

Tab. 3 Development of the content of sulphur of oxidising microorganisms in borehole S-24. Only the most abundant OTU are shown. The numbers below each sample indicate the amounts of OTU detected *in the given sample, the colour range indicates the amount of the given OTU in the sample (decrease in the order red-yellow-green)*

Borehole S-24	Water May 2018	Biofilm May 2018	Biofilm October 2018	Biofilm November 2018
Name	VZ31	VZ31a	VZ63	VZ110
Paracoccus pantotrophus	0	0	0	1
Roseococcus sp.	0	0	0	2
Sideroxydans sp.	8	0	0	0
Sulphuricurvum kujiense	102	45	21	76
Sulphuricurvum sp.	68	68	0	2
Sulphurisoma sediminicola	12	0	4	15
Sulphuritalea sp.	13	0	11	1
Thioalkalivibrio sp.	3	1	1	2
Thiobacillus sajanensis	37	14	3221	2907
Thiobacillus sajanensis	15	7	143	53
Thiobacillus sp.	1618	0	0	0
Thiothrix unzii	0	0	21	1984

Tab. 4 Development of the content of sulphur of oxidising microorganisms in borehole S-25. Only the most abundant OTU are shown. The numbers below each sample indicate the amounts of OTU detected in the given sample, the colour range indicates the amount of the given OTU in the sample (decrease in the order red-yellow-green)

Borehole S-25	Water May 2018	Biofilm May 2018	Water October 2018	Biofilm October 2018	Biofilm November 2018	Packer-filter January 2019
Name	VZ28	VZ28a	VZ60	VZ59	VZ111	VZ108
Paracoccus pantotrophus	0	0	0	0	0	52
Sideroxydans sp.	0	0	0	0	0	6
Sulphuricurvum kujiense	1995	2775	1797	1092	1669	29
Sulphuricurvum sp.	1853	2168	3	718	634	0
Sulphurisoma sediminicola	0	0	0	0	0	5
Sulphuritalea sp.	16	4	3	11	7	0
Thioalkalivibrio sp.	0	0	0	0	0	7
Thiobacillus sajanensis	0	0	12	47	120	29
Thiobacillus sajanensis	0	0	2	6	2	0
Thiothrix fructosivorans	0	0	0	0	0	5
Thiothrix unzii	0	0	0	0	0	5

Tab. 5 Development of the content of sulphur of oxidising microorganisms in borehole S-26. Only the most abundant OTU are shown. The numbers below each sample indicate the amounts of OTU detected in the given sample, the colour range indicates the amount of the given OTU in the sample (decrease in the order red-yellow-green)

Borehole S-26	Water May 2018	Biofilm May 2018 collected with a spatula	Biofilm May 2018 collected with a scalpel	Water October 2018	Biofilm October 2018	Biofilm November 2018	Water January 2019
Name	VZ20	VZ20a	VZ29	VZ57	VZ58	VZ112	VZ101
Paracoccus pantotrophus	0	0	0	4	0	1	899
Roseococcus sp.	0	0	0	1	0	0	1
Sideroxydans sp.	0	0	0	19	0	0	11
Sulphuricurvum kujiense	2169	1690	652	898	40	25	74
Sulphuricurvum sp.	279	109	99	5	1	0	0
Sulphurisoma sediminicola	0	0	0	0	1	4	1
Sulphuritalea sp.	5	0	2	1	11	32	0
Sulphuritalea sp.	0	0	0	1	0	0	0
Thioalkalivibrio sp.	0	0	0	2	0	1	3
Thiobacillus sajanensis	1389	1244	1291	2223	1631	3387	311
Thiobacillus sajanensis	49	59	488	250	329	32	16
Thiothrix fructosivorans	0	0	0	282	0	0	1258
Thiothrix unzii	0	0	0	1	0	0	0

- • ***Microbial oxidation of hydrogen***

Hydrogen oxidising microorganisms predominate in boreholes S-25 and S-26 on the 12th level and in borehole 4913 on the 24th level. These are represented by the bacteria *Hydogenophaga sp.* and *Sulphuricurvum kujiense*. Hydrogenoral microorganisms are present to a lesser extent in boreholes S-22, S-24, S-2, in an unmarked borehole below BK-38 (VZ76), in seepages BK-06, BK-38, BK-07 in microbial mats in boreholes BK-06B and BR-49, in water on the mine floor on the 12th level (VZ23), and on the 24th level in microbial mats in boreholes 4909, v-4588, and 4769. In addition to the two microorganisms listed above, they include *Nitrospira moscoviensis, Sulfuricurvum sp., Sulphurisoma sediminicola,* and *Sulphuritalea sp.*

- ***Microbial oxidation of methane***

The following bacteria are present in the Rožná mine that are the microorganisms capable of oxidising methane and possibly other low molecular weight hydrocarbon derivatives such as methanol or acetate: *Methylocystis rosea, Methylobacillus sp., Ferribacterium limneticum, Methylotenera versatilis, Methylomagnum ishisawai* and *Methylocapsa palsarum*. Interestingly, methylotrophic microorganisms predominate in biofilms on the 24th level, while on the 12th level they are not so common. On the 24th level, they are abundant in microbial mats in boreholes 4922, v-4588, 4674, and in the rill on the mine floor in the black fibre of a biogenic origin (VZ18). On the 12th level they are more common only in boreholes S-23 and S-1.

- ***Microbial oxidation of reduced nitrogen forms***

The ability to oxidise reduced forms of nitrogen (ammonia, nitrites), as well as other metabolisms, is commonly found in the microbial communities of the 12th and 24th levels. On the 12th level, these microorganisms are abundant in boreholes S-22, S-20, S-26, BK-06, BK-23, BK-07, BK-15, BK-29/33, S-2, BK-31, BK-38, BK-06B, BK-32 and in the water on the mine floor containing soft cauliflower-like formations and dark red crusts (VZ25 and VZ23). On the 24th level, nitrifying bacteria are abundant in boreholes v-4588, GR-07, 4680, the borehole near RVPŠ 124-21, 4674 and in the water on the mine floor with soft cauliflower-like formations (VZ14). Microorganisms oxidising either ammonia or nitrites at the Rožná mine include *Nitrospira moscoviensis, Nitrospira sp., Nitrosopumilus sp., Gemmata sp., Nitrososphaera sp., Nitrotoga sp.* and *Paracoccus pantrophus.*

- ***Microbial oxidation of organic substances***

Microbial oxidation of organic substances is common in all of the sampling sites in the Rožná mine. This phenomenon is common in all ecosystems, including groundwater, and the Rožná mine is no exception in this respect. Therefore, it is more useful to mention places where the microbial oxidation of organic substances is less important than the oxidation of inorganic compounds, rather than dealing with sites where the method of obtaining energy from respiration or fermentation of organic substances is dominant for microorganisms. Interestingly, these are generally boreholes where the oxidation of iron by *Gallionella sp*. dominates, and which is often accompanied by the presence of *Nitrosopumilus sp.*, which specialises in the oxidation of ammonia. *Gallionella sp.* bacteria grow in oligotrophic environments. In the Rožná mine, these places include: borehole S-20, BR-52, BK-23, BK-32 and the microbial mats near BK-06, BK-23, BK-06B and borehole GR-07 on the 24th level.

Microorganisms from the point of view of the electron acceptors used

- ***Microbial respiration of oxygen***

The microbial respiration of oxygen was determined in all of the examined samples. It can be said that this type of respiration was the lowest in the water from BK-35, in the orange streamer on discharge BR-50, in three microbial mats from the 24th level (boreholes 4680, 4517 and the borehole on RVPŠ 124-21) and on the 24th level in the rill with the microbial mat (VZ14).

- ***Microbial respiration of nitrates***

Nitrate respiration is most often present in microorganisms that respire oxygen and is used when oxygen is expended. This type of metabolism is very common in environments where oxygen concentrations fluctuate. In the Rožná Mine, the ability to respirate nitrates is present mainly in microorganisms in the water and biofilm from boreholes S-26, and S-25, where it was repeatedly detected. In these boreholes, the bacteria oxidising hydrogen and reduced forms of sulphur include *Hydrogenophaga sp., Hydrogenophaga aquatica, Sulfuricurvum kujiense,* and *Sulfuricurvum sp.* Other places with significant microbial respiration of nitrates are mainly biofilms, i.e. in the borehole S-22, on the 12th level (VZ23), and in boreholes 4913, 4922, v-4588, and 4680 on the 24th level.

- ***Microbial respiration of iron (iron reduction)***

Microbial iron reduction in the Rožná mine is a rather exceptional method of respiration. Iron-reducing microorganisms are common on the 12th level only in borehole S-1 in the Bukov URF, where they were detected repeatedly in October and November 2018. The water in the Bukov URF have a pH of between 6 and 10, and oxidised iron (Fe^{3+}) is most likely present in the solid phase (e.g., hydroxides, iron oxyhydroxides). These mineral products of weathering iron can serve as terminal electron acceptors from microbial respiration in the local rocks (in the case of both borehole S-1 and in the pipe leading from the borehole). Outside the Bukov URF, these microorganisms were noteworthy only in two samples on the 24th level - the ochre microbial mat near borehole 4922 and in the white microbial mat in the rill on the mine floor. The most common representatives of iron reduction in the Rožná mine are the heterotrophic bacteria *Ferribacterium limneticum*, which also respire nitrates, and fumarate and *Metallibacterium scheffleri*, which use iron only if there is insufficient oxygen in the environment. While the first of the mentioned microorganisms is typical in borehole S-1, the second is predominant in the other two samples from the 24th level.

- ***Microbial respiration of sulphates, thiosulphates and elemental sulphur***

Microbial reduction of sulphur compounds is even less significant than iron reduction in the studied areas of the Rožná Mine. *Alkaliphilus sp.,* which oxidises organic substances using elemental sulphur, thiosulfate or fumarate, is practically the only more abundant microorganism that has the ability to reduce oxidised forms of sulphur. It is not strictly dominant in any area, and is only a minor part of the microbial communities in the water from BR-51 and BR-52, where it is also part of the orange streamer forming on the wall below the discharge. It is also present in the microbial mat in borehole 4680.

- ***Microbial fermentation of organic substances***

Microbial fermentation of organic substances in the microbial communities in the Rožná Mine is also a rather unique way of shedding electrons in the oxidation of reduced compounds. However, it is more common than the reduction of iron and sulphur compounds. This metabolism is typical for biofilms and sediments on the mine floor in the mouths of boreholes, i.e. in an environment where oxygen is consumed as a result of the accumulation of organic substances and their degradation, so that it no longer penetrates into the lower sediment/biofilm layers and there is room for fermenting microorganisms. They are the most abundant in the biofilm in boreholes v-4689 and 4769 on the 24th level, where they are present in the form of facultative anaerobic bacterium *Caldilinea sp.,* which ferments only in the case of a lack of oxygen. Other areas with more numerous fermenting microorganisms (especially the anaerobes *Levilinea sp. and Terrimicrobium sacchariphylum*) are the microbial mats in borehole 4674, the mat on the 24th level in the rill with ochre, cauliflower-like growths (VZ14), the biofilm in borehole S-26 and the black sediment in borehole 4513. In water, fermenting bacteria are more significant only in boreholes S-21 and BK-15, where they are mainly the facultative anaerobe *Planctomyces sp.*

4.2.2 Development of microbial diversity in the Bukov URF

Indirect gradient analysis PCA was performed on all of the samples from the Bukov URF to identify the main features of microbial diversity. Fig. 16 shows that the samples taken at the same place are more similar in microbial content than samples taken at the same time. Therefore, there are no significant changes in microbial composition from the samples collected between October and November. The difference is only seen in sample from the borehole below BK-35 taken between the autumn and January sampling. There are also three time points from the sample from the borehole below BK-38, but here the January sampling does not differ from the autumn sampling. There is no data available for the other samples from the January sampling due to recurrent problems during the DNA sequencing in these samples. The change in diversity between the two samples at different times is negligible, but there is a noticeable difference between the microbial composition of water and the microbial mat, even though the samples were collected from the same borehole. This phenomenon is quite common because of the significant changes in the conditions of biofilm formation, i.e. the accumulation of organic substances and other metabolic products, the emergence of microaerophilic and anaerobic microenvironments in the biofilm, which leads to diversification in the microbial community.

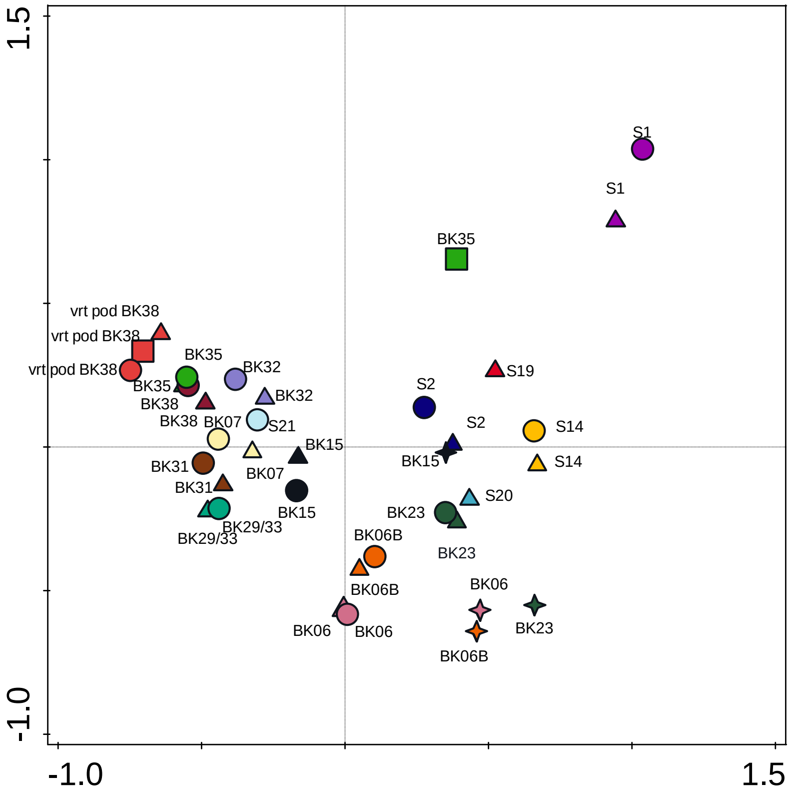

Fig. 16 Microbial diversity of water and microbial mats in the Bukov URF. Microbial diversity is represented by the graph of PCA analysis in the Canoco program. The individual symbols represent individual samples. The contents of the samples are the most abundant OTU. The closer the samples

are in the graph, the more similar their microbial composition. Samples from the same borehole/seepage are marked with the same colour and place name. Circle - water sampling in October, triangle - water sampling in November, square - water sampling in January, star - microbial mat sampling in October.

4.2.3 Results of the qPCR analysis

The results of the qPCR analysis for all of the three analysed markers are shown in Tab. 6.

Total bacterial biomass was found in all of the samples in different abundances and depended mainly on the type of sample (microbial growth vs. water).

Sulphur reducing bacteria were detected using the apsA gene in 48 samples, but were not significantly abundant in any of the analysed samples, which was consistent with the sequencing analysis. SRBs were found abundantly in samples taken using the passive sampler (compared to the previous sampling using Sterivex filters to capture the situation prior to installing the packers), which indicated growth of a reductive environment in the boreholes. Other than these samples, SRB were abundantly represented in samples 25 and 59 (both microbial growths) and in the water from borehole S19 (sample 82).

Denitrifying bacteria were detected in 84 samples using the nirK gene. Although the samples differed in terms of abundance, they were not extremely abundant in any of the samples studied. The samples with the highest proportion of NRB were the microbial growths in samples 30, 44 and 102.

Tab. 6 Results the qPCR analysis. SRB: sulphate reducing bacteria, NRB: denitrifying bacteria, PV: passive sampler, +++: abundant bacteria of the given group, ++: medium amounts of bacteria in the given group, +: small amounts of bacteria in the given group, -: undetected bacteria of the given group.

DNA sample No.	Name of the sampling point (e.g. borehole, seepage labelling)	Sampling date	Sample type	Total bacterial biomass (16S rRNA)	SRB (apsA)	NRB (nirK)
1	BR-21	22/05/18	water	+	+	+
2	borehole 4470	22/05/18	growth	++	+	+
2a	borehole 4470	22/05/18	growth	++	-	+
3	borehole 4513	22/05/18	growth	+	-	+
4	borehole 4517	22/05/18	growth	+	+	-
5	borehole 4674	22/05/18	growth	+++	+	++
6	borehole v-4588, RVPŠ 124-13	22/05/18	growth	++	-	+
6a	borehole v-4588, RVPŠ 124-13	22/05/18	growth	++	-	-
7	borehole 4680	22/05/18	growth	+	-	-
8	borehole v-4689, RZPŠ 124-19	22/05/18	growth	+++	-	-
9	borehole RVPŠ 124-21	22/05/18	growth	++	+	+
9a	borehole RVPŠ 124-21	22/05/18	growth	+	-	+
10	borehole 4769	22/05/18	growth	+++	++	+

11	borehole 4922, RVPŠ 124-29	22/05/18	growth	++	-	++
11a	borehole 4922, RVPŠ 124-29	22/05/18	growth	+	+	+
12	borehole 4905	22/05/18	growth	+	-	-
13	borehole 4909	22/05/18	growth	++	-	+
14	mine floor 4m north of borehole 4909	22/05/18	growth	+++	+++	++
15	mine floor between boreholes 4907 and 4908	22/05/18	growth	++	-	+
16	mine floor between boreholes 4909 and 4910	22/05/18	growth	++	+	+
17	mine floor 4m north of borehole 4910	22/05/18	growth	+	-	-
18	mine floor 4m north of borehole 4910	22/05/18	growth	++	-	+
19	borehole 4913, 61 - geotest	22/05/18	growth	++	-	+
20	borehole S-26	22/05/18	water	++	++	-
20a	borehole S-26	22/05/18	growth	++	+	-
21	discharge BR-49	23/05/18	growth	++	+	++
21a	discharge BR-49	23/05/18	growth	++	+	++
22	fracture near 12011	23/05/18	growth	+	-	-
23	mine floor near 12736	23/05/18	growth	+	+	+
24	BR-51, borehole 911	23/05/18	water	+	-	-
25	mine floor near 12052	23/05/18	growth	++	+++	++
26	BR-52, borehole on the western side of the corridor	23/05/18	water	+	-	-
26a	BR-52, borehole on the western side of the corridor	23/05/18	growth	+	-	-
26b	BR-52, borehole on the western side of the corridor	23/05/18	growth	++	-	-
27	BR-53, borehole on the western side of the corridor	23/05/18	water	++	-	+
27a	BR-53, borehole on the western side of the corridor	23/05/18	growth	+++	+	++
28	borehole S-25	23/05/18	water	+++	+	++
28a	borehole S-25	23/05/18	growth	+++	+	+
29	borehole S-26	23/05/18	growth	++	-	-
30	borehole S-22	23/05/18	growth	+++	++	+++
31	borehole S-24	23/05/18	water	+	-	-
31a	borehole S-24	23/05/18	growth	++	+	+
32	discharge BR-47	23/05/18	growth	+	-	-
33	discharge BR-50	23/05/18	growth	++	+	++

34	GR-06 discharge from the borehole east of $RZ1_{24}$-113	04/04/18	water	++	-	+
35	GR-07 discharge from the borehole in $RZ1_{24}$-117	04/04/18	water	++	+	+
36	BK23	09/10/18	water	+	-	-
37	BK23	09/10/18	growth	++	-	++
38	BK06	09/10/18	water	+	-	-
39	BK06B	09/10/18	water	+	-	-
40	BK06B	09/10/18	growth	++	-	-
41	BK07	09/10/18	water	+	-	++
42	BK29/33	09/10/18	water	+	-	-
43	BK15	09/10/18	water	+	-	+
44	BK15	09/10/18	growth	++	-	+++
45	borehole S1	09/10/18	water	+	-	-
46	borehole S2	09/10/18	water	+	-	+
47	BK31	09/10/18	water	++	-	-
48	BK32	09/10/18	water	++	-	-
49	unnamed borehole in the wall below BK38	09/10/18	water	+++	-	+
50	borehole S20	09/10/18	water	+	-	+
51	borehole S14	09/10/18	water	+	-	-
52	borehole S21	10/10/18	water	++	-	+
53	BK35	10/10/18	water	++	-	+
54	BK38	10/10/18	water	++	-	+
55	opposite BK06	10/10/18	growth	++	+	+
56	borehole S23	10/10/18	water	+	-	-
57	borehole S26	10/10/18	water	+	+	-
58	borehole S26	10/10/18	growth	+++	+	+
59	borehole S25	10/10/18	growth	+++	+++	+
60	borehole S25	10/10/18	water	++	+	++
61	borehole S22	10/10/18	growth	++	+	+++
62	borehole S24	10/10/18	water	++	+	+++
63	borehole S24	10/10/18	growth	+++	-	++
64	BR-52	10/10/18	water	+	-	-
65	BR-52	10/10/18	growth	+++	-	+
66	BK23	21/11/18	water	+	+	+
67	BK06	21/11/18	water	++	-	-
68	BK06B	21/11/18	water	++	+	-
69	BK07	21/11/18	water	++	-	+
70	BK29/33	21/11/18	water	++	+	+
71	BK15	21/11/18	water	+	+	+
72	borehole S1	21/11/18	water	+	-	-
73	borehole S2	21/11/18	water	++	-	-
74	BK31	21/11/18	water	++	+	+++
75	BK32	21/11/18	water	++	-	+

76	unnamed borehole in the wall below BK38	21/11/18	water	+	-	+
77	borehole S20	21/11/18	water	+	-	+
78	borehole S14	21/11/18	water	++	-	+
79	borehole S21	21/11/18	water	++	-	+
80	BK35	21/11/18	water	+++	-	+
81	BK38	21/11/18	water	++	-	+
82	borehole S19	21/11/18	water	+++	+++	+
83	BK23	09/01/19	water	+	-	-
84	BK06	09/01/19	water	++	-	-
85	BK06B	09/01/19	water	+	-	-
86	BK07	09/01/19	water	++	-	+
87	BK29/33	09/01/19	water	++	-	+
88	BK15	09/01/19	water	++	-	+
89	borehole S1	09/01/19	water	++	+	+
90	borehole S2	09/01/19	water	++	+++	+
91	BK31	09/01/19	water	++	-	+
92	BK32	09/01/19	water	++	-	+++
93	unnamed borehole in the wall below BK38	09/01/19	water	++	-	-
94	borehole S20	09/01/19	water	+	-	-
95	borehole S14	09/01/19	water	+	-	++
96	borehole S21	09/01/19	water	++	-	++
97	BK35	09/01/19	water	++	-	+
98	BK38	09/01/19	water	++	-	+
99	borehole S19	09/01/19	water	++	+++	+++
100	borehole S23	15/11/18	water	+	+	+
101	borehole S26	15/11/18	water	++	++	+++
102	borehole S22	15/11/18	growth	+++	+	+++
103	borehole S1	15/11/18	water (GW)	++	+++	-
105	borehole S22	09/01/19	water (GW)	++	++	+++
106	borehole S23	09/01/19	water (GW)	++	+	+
107	borehole S24	09/01/19	water (GW)	+++	+++	++
108	borehole S25	09/01/19	water (GW)	+	+++	+
109	borehole S23	15/11/18	growth	++	+	+++
110	borehole S24	15/11/18	growth	+++	-	+++
111	borehole S25	15/11/18	growth	+++	+++	++
112	borehole S26	15/11/18	growth	+++	+++	++

5 Cultivation microbiological analysis

5.1 Introduction

Samples taken during the project were analysed by cultivation methods to formulise a comprehensive description of the microbial colonisation of microorganisms cultivable by defined procedures that are minimally selective in the determination. The total number of cultivable microorganisms was determined as the number of heterotrophic microorganisms under aerobic and anaerobic conditions. To determine denitrifying microorganisms, a nitrate anion was used as the terminal electron acceptor within the culture medium. Sulphur reducing bacteria were determined using API media. Lovley agar was used to determine the microorganisms that reduce ferric ions through respiration. Finally, methanogenic microorganisms were determined in the samples by a semi-quantitative method in vials with an inert atmosphere. Sulphide oxidising bacteria were represented by lithotrophic microorganisms and DSMZ medium 35 was used to quantify them.

5.2 Materials and methods

5.2.1 Processing of samples

All of the analysed samples were supplied in the form of groundwater samples taken aseptically and intact. Immediately after delivery, the samples were diluted to a defined concentration using sterile saline (0.9% sodium chloride solution in distilled water) using a decimal dilution method (Goldman and Green, 2008) and placed in sterile microbiological test tubes with a screw cap. Standardised spreading and potting methods were used to distribute the diluted samples on the agar surface (Goldman and Green, 2008). Spreading with a vaccination stick was performed on dry agar plates, and the relevant agar, cooled to approximately 40-45°C, was used for pouring. The below table (Tab. 7) summarises the methods and procedures used for the specific analysis.

Tab. 7 Summary of the processing of samples

Type of analysis	Method of placing the sample into the agar	Type of cultivation system	Cultivation conditions
aerobic heterotrophic bacteria	1 ml smear	Petri dish	cultivation box
anaerobic heterotrophic bacteria	1 ml pouring	Petri dish	jar box
denitrifying bacteria	1 ml pouring	Petri dish	jar box
iron reducing bacteria	1 ml pouring	Petri dish	jar box
sulphate reducing bacteria	1 ml pouring	Petri dish	jar box
methanogenic microorganisms	syringe 0.1 ml puncture through the septum	Petri dish	vials (carbon

			dioxide and hydrogen)
sulphide oxidising bacteria	1 ml smear	Petri dish	cultivation box

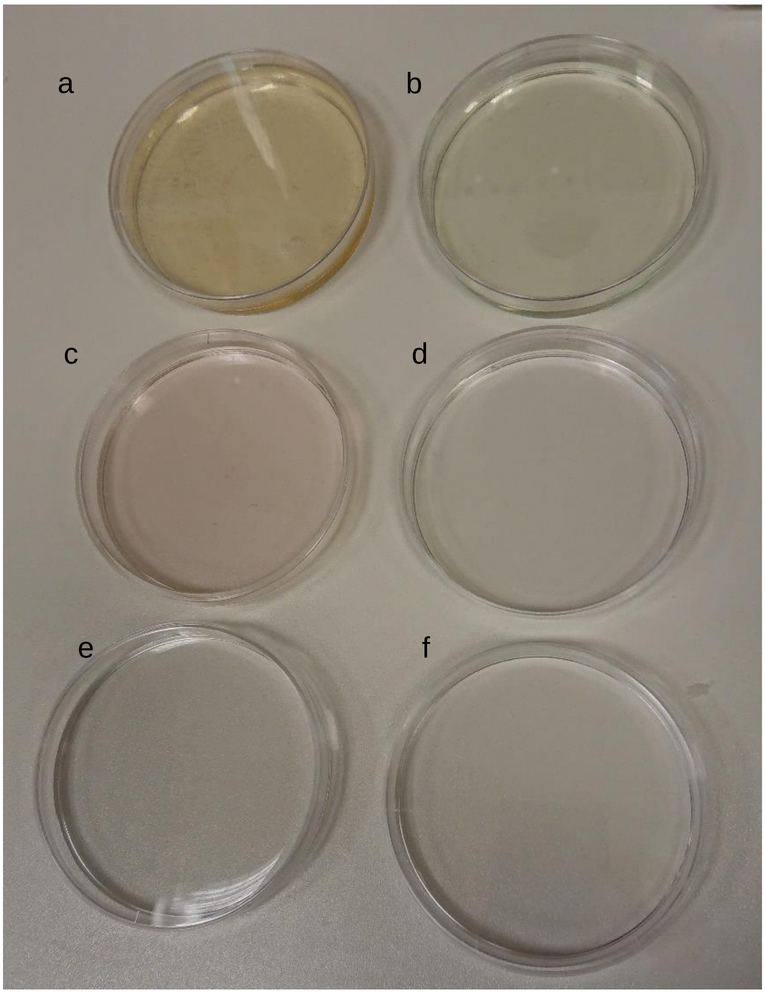

Fig. 17 Basic culture media set: plate count agar (a), R1A (b), FWA-FeIII (c), API (d), agar for methanogens (e) and DSMZ medium 35 (f)

In the case of the analysis of methanogenic microorganisms, a strict anaerobic technique had to be followed, which was ensured by handling the samples with a syringe and vials with a septum. Liquid media were vented by boiling and adding an antioxidant, the absence of oxygen was controlled by resazurin. The resazurin test was used to control the anaerobic environment (resazurin reacts very sensitively to an increase in Eh values). The resazurin test is commonly used in microbiology for the cultivation of anaerobic organisms (e.g., Proctor 1997) as a reliable way of confirming anaerobic conditions.

5.2.2 Culture analysis on agar plates

The analysis focused on various functional groups of microorganisms. Except for methanogenic populations, the remaining culture analyses were performed in a Petri dish that was placed in a thermostat at a constant temperature (15°C). Due to the fact that the nature of the samples analysed is indicative of a significantly oligotrophic system (lack of an easily usable source of carbon and energy such as carbohydrates, lipids or proteins), it is highly likely that in many cases the adaptation of the studied microorganisms may take considerably longer than usual in reference strains. For the purpose of suppressing false positive or negative results, the cultivation was performed over a longer period of time and with more readings of the growth in the form of the created colonies. In order to prevent the agar plates from drying out, a source of sterile water was placed in the culture medium throughout the cultivation to maintain a stable moisture level in the environment. The names, composition and origin of the culture media used (Tab. 8) are completed by a graphical representation of the plates (Fig. 17) prior to inoculation.

Tab. 8 Solidified culture media used

Name of media	Composition	Analysis	Source
Plate count agar	agar 9.0 g/L, dextrose, 1.0 g/L, tryptone, 5.0 g/L, yeast extract, 2.5 g/L	heterotrophic microorganisms	Sigma Aldrich
R2A	in 1 L distilled water 0.5 g KNO_3, 0.5 g yeast extract, 0.5 g peptone, 0.5 g glucose, 0.5 g starch, 0.3 g sodium pyruvate, 0.3 g K_2HPO_4, 0.05 g MgSO4. 7H2O and 10 ml trace element solution (composed of 10 ml HCl, 1.5g $FeCl_2$. $4H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2$. $4H_2O$, 6 mg H_3BO_3, 190 mg $CoCl_2$. $6H_2O$, 2 mg $CuCl_2$. $2H_2O$, 24 mg $NiCl_2$. $6H_2O$, 36 mg Na_2MoO_4. $2H_2O$), 15g agar	denitrifying bacteria	Massa, Carusso, Trovatelli, Tosques (1998)
FWA-Fe(III)	in 1 L of distilled water 2.5 g $NaHCO_3$; 0.1 g $CaCl_2$ $2H_20$, 0.1 g KCl, 1,5 g NH_4Cl; 0.6 g NaH_2PO_4 H_20, 6.8 g $NaCH_3COO$ and 200 mmol amorphous ferric ions/L and 15 g agar	iron reducing bacteria	Lovley a Phillips (1988)
API	agar, 14 g/L, ascorbic acid, 0.1 g/L, disodium phosphate, 0.01 g/L, ferrous ammonium sulphate, 0.1 g/L, magnesium sulphide, 0.2 g/L	sulphate reducing bacteria	Sigma Aldrich
Agar for methanogens	NH_4Cl, 1 g/L; NaCl, 0.6 g/L; $NaHCO_3$, 5 v; KH_2PO_4, 0.3 g/L; K_2HPO_4, 0.3 g/L; $MgCl_2.6H_2O$, 0.16 g/L; $CaCl_2.2H_2O$, 0.009 g/L; resazurin 0.1% solution, 1 mL; cysteine, HCl and $Na_2S.9H_2O$ and 15 g agar	methanogenic microorganisms	Wolfe (2011)

DSMZ medium 35	NH_4Cl 0.10 g KH_2PO_4 3.00 g $MgCl_2.6H2O$ 0.10 g $CaCl_2.2H_2O$ 0.14 g sulphur powder 10.00 g distilled water 1000.00 ml	sulphide oxidising bacteria	DSMZ

5.2.3 Cultivation of methanogenic microorganisms

Cultivation of methanogenic microorganisms performed according to the methodology (Wolfe 2011), simplified to the environment of 2 mL vials, into which 0.5 mL of methanogen agar was added (Tab. 8) and after solidification and closure of the septum an inert atmosphere of the carbon dioxide-hydrogen mixture was formed (80:20). Inoculation was performed using a syringe, the grown biomass was visualised using TTC.

5.2.4 Results

As was already mentioned (chapter ***Chyba! Nenalezen zdroj odkazů.***), due to the highly o ligotrophic nature of the samples, the cultivation was performed until the number of colonies formed remained at constant values. In the summary (Tab. 9), this fact is taken into account by specifying the length of cultivation. The semi-quantitative character of the determination of methanogenic microorganisms is manifested by determining only the order of concentrations expressed as CFU in mL of the original sample. In other cases, the number of microorganisms in the sample was determined by subtracting the growth on the plate and converting it to the original sample.

Tab. 9 Results of the microbiological analysis expressed as CFU in mL (TPC – technically pure culture)

Sample	Analysis	2 days	7 days	21 days	42 days	70 days	TPC
BK 06	aerobic heterotrophic bacteria	8.3E+03					1
BK 06 B		7.8E+03					2
BK 30		7.1E+03					2
BK 26		6.2E+03					1
BK 27		1.9E+03					3
BK 31		2.6E+03					1
BK 19		9.9E+03					2
BK 06	anaerobic heterotrophic bacteria			1.1E+02			2
BK 06 B			5.2E+02				1
BK 30				9.7E+02			1
BK 26			7.9E+02				1
BK 27				4.2E+02			2
BK 31				7.7E+02			1
BK 19				5.4E+03			2

BK 06	denitrifying bacteria		7.5E+01				1
BK 06 B			2.0E+02				1
BK 30		4.6E+01					1
BK 26		7.5E+01					1
BK 27			3.6E+02				1
BK 31			4.1E+02				2
BK 19			1.3E+02				1
BK 06	iron reducing bacteria			3.8E+01			1
BK 06 B			5.4E+01				1
BK 30				2.9E+00			1
BK 26				4.7E+00			1
BK 27					2.1E+01		1
BK 31				9.0E-01			1
BK 19				1.3E+00			1
BK 06	sulphate reducing bacteria					0	
BK 06 B						0	
BK 30						0	
BK 26						0	
BK 27						0	
BK 31						0	
BK 19						0	
BK 06	methanogenic microorganisms				1.0E+01		
BK 06 B					0		
BK 30					0		
BK 26					0		
BK 27					1.0E+01		
BK 31					1.0E+01		
BK 19					0		
BK 06	sulphide oxidising bacteria		2.2E+01				2
BK 06 B			3.7E+02				3
BK 30			2.0E+03				3
BK 26			1.2E+03				2
BK 27			4.3E+03				1
BK 31			1.0E+02				2
BK 19			3.3E+03				2

The last column expresses the number of isolated technically pure cultures (cultures that originated from a single colony that is isolated, bounded and sufficiently distant). This number also expresses the number of available biological agent isolates that have been isolated from the natural system and selected morphologically.

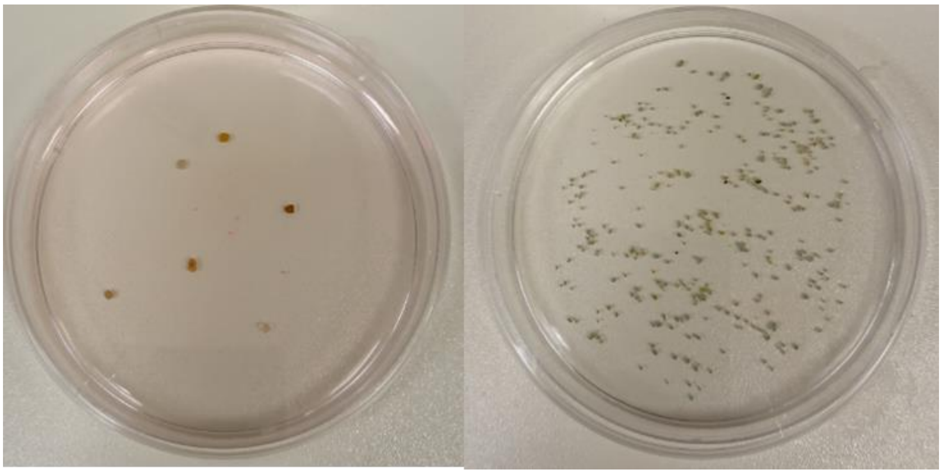

Fig. 18 Examples of cultivated microorganisms, left IRB, right SOB from sample BK 30

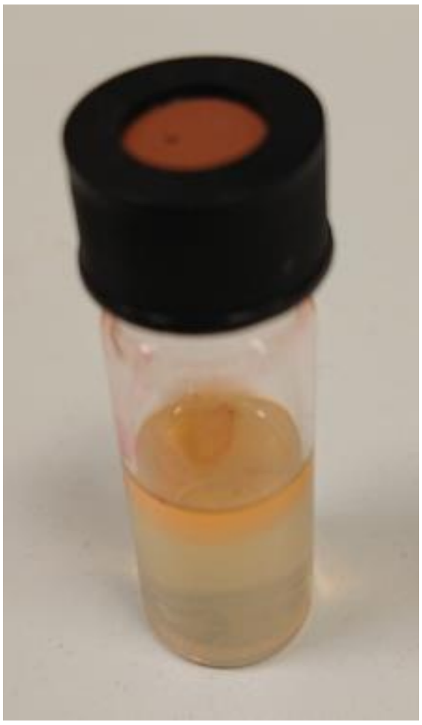

Fig. 19 Positive reaction during the cultivation of methanogenic microorganisms

5.2.5 Conclusion

The profiles of the present microorganisms were determined in seven analysed samples with an emphasis on their functional characteristics and different times of growth. The most prominent group is that of aerobic heterotrophic bacteria, which were found in similar patterns in all of the studied samples. They also showed the greatest degree of heterogeneity, i.e. the highest number of isolable biological agents. The presence of anaerobic heterotrophic microorganisms ranged around an order of magnitude lower. Small amounts of denitrifying bacteria were present in the samples; however, the possible facultative nature of their metabolism, such as their ability to switch between aerobic respiration and denitrification, should be taken into account for this parameter. In terms of other metabolic types where a substance other than oxygen or nitrate figured in the position of terminal electron acceptor, the presence of iron-reducing bacteria was confirmed (Fig. 18 left), from which one morphologically identical isolate found in all of the samples was isolated. On the contrary, it was again not possible prove the presence of bacteria with the ability to transfer electrons in the respiratory process to sulphates that could be cultivated. Nevertheless, in the framework of the metabolism of sulphur, the high rate of bacteria that oxidise reduced sulphur compounds is noteworthy (Fig. 18 right). After cultivation for 42 days, the presence of cultivable methanogenic microorganisms was confirmed in three samples (Fig. 19).

5.3 Comparison of the results of the cultivation methods and molecular methods

Seven water samples from seven different boreholes in the Bukov URF were processed using cultivation methods. By analysing 16S rDNA amplicons, four samples were processed from these seven water samples. These were samples BK-06, BK-06B, BK-27 (S-2), and BK-31. The results of the two methods are quite different, mainly due to the low cultivability of oligotrophic autotrophic microorganisms, resulting in an overestimation of the eutrophic heterotrophic aerobic metabolic groups during the cultivation. Molecular methods show that in samples BK-06 and BK-31, ammonia, iron and sulphur oxidisers dominate, i.e. aerobic autotrophs and not aerobic heterotrophs (note that the oxidation of ammonia and iron was not tested using the cultivation methods). In terms of the electron acceptors used, molecular methods show a complete dominance of aerobic microorganisms, many of which have the potential to use nitrates as TAE. Fermenting, sulphate-reducing and iron-reducing microorganisms play an absolutely unimportant role here (in the order of per mille of the total number of amplicons). Methanogenesis in these four water samples was not detected at all in the analysis of the most abundant OTU. In contrast, the cultivation methods were able to detect a surprisingly large amount of iron and methanogen reducers. This difference can be explained by the fact that, in theory, even a small number of inactive cells in the original environment is sufficient for successful cultivation, which only begin to grow when suitable conditions (seeding on a given medium) occur. The cultivation methods are highly selective and show the presence of microorganisms cultivable in a given medium, from which it is not possible to determine the relative representation of individual metabolic groups in the original community. Molecular methods serve this purpose better.

6 Effects of anthropogenic activities on microbial communities

An underground environment such as a mine is created through human activity. The rock environment is influenced by anthropogenic activity and this influence is reflected in the character of microbial communities studied in the mining works. This is the case with the Rožná mine. Microorganisms need energy to grow, when they are underground, where phototrophy is not possible, they mainly acquire it chemotrophically, i.e. through oxidation-reduction reactions, where electrons are transferred from donors to electron acceptors and the released energy is stored in ATP molecules. The most energy-efficient method is to transfer electrons to oxygen, i.e. aerobic respiration. As the mine works are ventilated, there is sufficient oxygen in the mine corridors to be used by the microorganisms for aerobic respiration. In the Rožná mine, such a type of metabolism clearly dominates in the seepages and boreholes from which the samples were taken in the immediate vicinity of the discharges from the wall, i.e. without a long flow through the mine corridor. It is important to note that oxygen from the mine corridors penetrates even to the deep boreholes and that very small concentrations of oxygen are sufficient for the growth of aerobic respiratory microorganisms, precisely due to it being an energy-efficient reaction. The occurrence of oxygen in these environments promotes the microbial aerobic oxidation of sulphur compounds, iron, nitrogen compounds, hydrogen methane, or other organic substances, and on the contrary, suppresses anaerobic metabolisms such as sulphate respiration, iron reduction, methanogenesis (not detected by the analysis of the most abundant microorganisms) or fermentation. The largest number of obligate anaerobes was typically found in biofilms, where in the lower micro-layers there is oxygen consumed by microorganisms growing in the higher layers of the biofilm (e.g. the biofilm in boreholes S-26, S-22 or several growths taken on the 24th level - boreholes 4922, 4674 and many others). The proportion of anaerobes in the biofilms growing in ventilated mine corridors is not determined by the location of the sampling site (depth below the surface), but rather by its thickness, which positively correlates with the rate of anoxia in its interior. It is therefore more interesting to look at the water samples that can be assumed to carry the anaerobic microorganisms from deeper anoxic areas in the rock. The highest relative representation of sequences belonging to obligate anaerobes was a maximum of one percent of the total proportion of the detected sequences. This relates mainly to borehole S-1, which has long been fitted with a packer and tap and borehole discharges are measured, where 7 % of the sequences belong to the obligate anaerobe of *Ferribacterium limneticum*, which uses fumarate, nitrates or ferric ions as TAE. Another example may be boreholes and seepages BR-21, BR-51, BR-52, and BR-53 with 3 %, 4 %, 2 % and 1.2 % obligate anaerobic sequences, respectively. These water samples contained either *Levilinea sp.*, which ferments organic substances, or *Alkaliphilus sp.,* which respires with the help of sulphur, thiosulfate or fumarate. It can be assumed that obligate anaerobic metabolisms are common at a greater distance from the mine corridors in the rock massive and would prevail just as well if the mine was closed and oxygen subsequently lost in the Rožná mine.

7 Proposal for further possible microbiological monitoring solutions

In relation to the results mentioned in the previous chapters, a proposal was made on how to best proceed with the monitoring of the microbiological activity in the study area. The high number of samples analysed and their heterogeneity made it possible to determine the basic limitations, which should be taken into account in the case of further activities. These limitations are primarily the influence of human activities on microbiological diversity leading to a high proportion of aerobic microorganisms and a low recovery rate of deep groundwater, which is typical of similar environments and significantly increases the risk of sample contamination.

As mentioned in the foregoing text, any microbial activity is potentially dangerous for the integrity of engineering barriers in the planned DGR. However, the following should be considered as the main hazardous processes: microbial-induced corrosion (especially sulphate-reducing and sulphur-oxidising bacteria, iron-reducing and iron-oxidising bacteria and nitrate-reducing bacteria) affecting the functional properties of bentonite (especially iron-reducing bacteria but also other groups), any microbial production or consumption of gases and microbial production of organic substances. All of the groups that have the potential to affect the safety of the DGR have been identified at the site and they will develop under the right conditions. Therefore, main objective of any further activities should be to find conditions that would significantly reduce the development of microbial activity.

Anaerobic conditions are planned in the potential deep geological repositories where abiotic and biotic forms of weathering of packaging materials are slower. Therefore, further attempts should be made to address microbial diversity so that the environment in which the experiments take place is as close as possible to the future real repository with anaerobic conditions. Chapter 3.2.1 demonstrated the metabolic diversity of microorganisms in deep geological repositories affected by aerobic conditions (anthropogenic activity - see Chapter 5). In the next step, we propose to perform experiments where an anaerobic regime will be established to support the growth of anaerobic microorganisms, i.e. those potentially present under the conditions of a deep geological repository. In this way, it will be possible to evaluate the microbial and metabolic diversity of microbial communities in groundwater as little affected as possible by the presence of oxygen.

In the event that further activities will be planned in the area of the Bukov URF or in the Rožná mine, where it would be appropriate to monitor the microbiological activity in the groundwater, it is necessary to consider the aim of the experiments and to adapt the method of sample collection, which is crucial for the results of the microbiological analysis, accordingly. In order to monitor changes in anaerobic experiments, it is essential to take water samples from vented boreholes with packers, where the content of oxygen is minimised and the highest possible reducing environment can be established. For the monitoring, it is advisable to choose boreholes with less fractured rock. Furthermore, it should be takin into consideration that the establishment of a microbiological balance takes a certain amount of time, which is hard to estimate, depending on the type of borehole and other circumstances (e.g. disturbances in the vicinity of the borehole), and therefore it is appropriate when planning the activities to allow for a certain time lapse after closing the borehole. Closure of the borehole is followed by gradual changes in the composition of the microbial community until a stable, so-called climax stage is reached. Only at this moment is it possible to observe changes in the microbial spectrum depending on a variable other than the time from the closure of the borehole. In addition to the microbiological sampling, for interpretation of results it should be good to monitor the hydrochemical parameters in the borehole (optimally in-situ). It would be ideal to extend the time of measuring the hydrochemical parameters to achieve steady-state conditions, especially in the case of measuring the oxidation-reduction potential (Eh). Furthermore, it would be appropriate to supplement the measurements with a hydrochemical analysis of groundwater from the filtrate. These additional analyses would help to reliably interpret the obtained data.

if possible, the best way to perform the sampling would be to use the new passive samplers presented in this report. The main reason is that the filter on which the microbial biomass is captured is inserted (and also extracted) in the laboratory in a sterile environment and thus does not contaminate the microorganisms found in the environment of the underground corridor. The samplers are fully sterilisable and allow larger volumes of water to be filtered. The results of our measurements show that in the case of boreholes with low recovery rates, it is best to filter at least three litres of water (preferably five litres or more) to obtain a sufficient about of DNA. However, it is time-consuming to filter such an amount of groundwater i.e. due to a limited connection to a 220 V source in the underground conditions of the site, so the sampling process will require further optimisation and cooperation with the Client. If, for any reason, it is not possible to use a passive sampler, then the use of Sterivex filters seems to be another suitable option. Sterivex filters have proven to be very successful in the case of samples where recovery rates are higher.

In the framework of this project, packers were installed on boreholes S-22 to S-26 for microbiological sampling and it would be appropriate to continue monitoring the changes in their microbial composition. Except for borehole S-22, the tubes from all of the other boreholes was only led out after the last sampling. Therefore, it is advisable to monitor any changes caused by borehole venting. Another advantage of continuing the monitoring of these boreholes would be to obtain an idea of how long it takes to establish a microbiological balance after closure of the borehole (which is particularly important for planning further experiments where evaluation of microbiological parameters is considered). Last but not least, the ongoing monitoring will enable the last problematic technical areas of the use of the sampling system to be solved so that it can be used routinely. These monitoring points can be expanded to include additional boreholes in the area of the Bukov URF, where sampling would be technically simpler (mainly due to the availability of a 220 V source) and the monitoring results (with respect to future experiments, which the Contractor expects to mainly take place in the URF) will be more relevant. The optimal sampling frequency for long-term monitoring is two to three months (shorter after installing a packer to a borehole).

Another parameter that very likely affects the composition of microbial communities is the pressure in the borehole. In order to study this effect, it is possible to use boreholes with installed packers in which the pressure increases after closure, and to monitor changes in the composition of the community depending on the pressure values (in-situ pressure experiments).

Last but not least, attention should also be paid to sessile microorganisms, which grow in the available substrate (mainly rock in the case of the studied area). Passive samplers with an inert sterile substrate placed in a closed borehole are used abroad in this case. Suitable substrates that can be placed into the sampler could be glass, sterilised crushed rock from the site, or nanofiber carriers whose large surface area allows for relatively rapid growth of a sufficient amount of biological material (although it is difficult to estimate how fast this process may take). Such carriers can be placed in both horizontal and vertical boreholes. For vertical boreholes, the samplers could be placed at different depths to monitor the effect of depth on microbial diversity. For example, borehole S-18 seems appropriate for this purpose.

8 Conclusion

The submitted final report summarises the results of a comprehensive microbiological screening performed at the Rožná mine and in the area of the Bukov URF between January 2018 and January 2019. A total of 129 samples were collected for microbiological analysis.

The investigation showed the occurrence of metabolically and phylogenetically diverse groups of microorganisms by a sequencing analysis of 16S rDNA amplicons and by cultivation methods. Furthermore, the samples were evaluated by quantitative PCR, which focussed on total bacterial biomass (gene for 16S rRNA), sulphate-reducing bacteria, and denitrifying bacteria. The analysis of amplicons was performed on both the 12th and 24th level and showed that there was no significant difference in the two levels in terms of microbial metabolisms. The only difference worth mentioning is the greater incidence of aerobic methylotrophic microorganisms on the 24th level. This may be due to the fact that most of the samples were taken from microbial mats (biofilms) and not from flowing water, which is lacking on the 24th level, or that the environment on the 24th level generally has less electron donors (again possibly due to lower flow rates), and therefore the oxidation of short methylated organic substances as products of the degradation of organic substances prevails. Another important finding is that microbial communities are significantly affected by anthropogenic activity in the researched environment of the Rožná mine, especially the mine ventilation and the presence of oxygen in the mine corridors and boreholes. As a result, in the Rožná Mine, obligate anaerobic microorganisms, which can otherwise be expected in natural environments at similar depths, are a rare exception. The most abundant functional groups are microorganisms capable of oxidising organic substances, iron compounds and sulphur.

A sampling system for microbiological analysis using passive samplers was developed and successfully used during the course of the project. These passive samplers were placed on the outlets of boreholes S-22, S-23, S-24, S-25 and S-26. They were used to capture the native species spectrum of microorganisms present in the anaerobic groundwater, to capture a greater amount of biomass, to minimise the impact of the aerobic environment of the tunnel/built repository and to eliminate the impact of the current anthropogenic interventions at the site. In-situ hydrochemical measurements were also performed on the boreholes where passive samplers were used.

Based on the findings made to-date, recommendations have been formulated for possible further activities involving microbiological analysis in the study area. In particular, these concern the establishment of microbiological conditions so that the growth of microorganisms that can be expected in a closed underground repository with a minimum oxygen content is promoted in the research environment. Therefore, the aim will be to focus on the diversity of anaerobic microbial groups.

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