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Detection of mycobacteria in the environment of the Moravian Karst (Bull Rock Cave and the relevant water catchment area): the impact of water sediment, earthworm castings and bat guano

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ABSTRACT: The presence of mycobacteria was studied in Bull Rock Cave ("Byci skala") and the water catchment area of Jedovnice Brook ("Jedovnicky potok") using direct microscopy after Ziehl-Neelsen (ZN) staining, culture examination and molecular techniques. Mycobacteria were detected in 47.1% of a total of 68 samples. The mycobacterial genes *hsp65* and *dnaA* were detected and sequenced in 37 (74.0%) out of the 50 cave environmental samples and in 10 (55.6%) out of the 18 samples of water catchment sediments. Nine species of slowly growing mycobacteria (*M. terrae*, *M. arupense*, *M. gordonae*, *M. lentiflavum*, *M. parascrofulaceum*, *M. parmense*, *M. saskatchewanense*, *M. simiae* and *M. xenopi*) and two subsp. (*M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*) were detected. Fourteen species of rapidly growing mycobacteria (*M. chelonae*, *M. chubuense*, *M. poriferae*, *M. flavescens*, *M. fortuitum*, *M. porcinum*, *M. rhodesiae*, *M. gilvum*, *M. goodii*, *M. peregrinum*, *M. mageritense*, *M. vanbaalenii*, *M. gadium* and *M. insubricum*) were detected. The highest mycobacterial presence was documented by ZN staining and/or culture examinations in earthworm castings and bat guano (73.3% positivity out of the 15 samples) in the cave environment and in the water sediments collected under the outflow from the wastewater treatment plants (77.8% positivity out of nine samples). The highest total organic carbon (TOC) was detected in wooden material and earthworm castings with pH values between 5.0 and 7.7 in the cave environment and in water sediments collected under the outflow from the wastewater treatment plants with pH between 5.8 and 7.0. It could be concluded that the karst cave environment with its running surface water contaminated with different microorganisms or chemical substances creates favourable conditions not only for animals (especially earthworms) but also for mycobacteria. This fact is also demonstrated by the presence of these mycobacteria in the cave environment mainly in earthworm castings and bat guano.

Keywords: bat faeces; environmentally derived mycobacteria; potentially pathogenic mycobacteria; ecology; geomycobacteriology; biospeleology; cave fauna; epidemiology

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List of abbreviations

AFR = acid-fast rods, **ESM** = environmental saprophytic mycobacteria, **IS** = insertion sequence, **L-J** = Lowenstein-Jensen, **LOI** = loss on ignition, **PPM** = potentially pathogenic mycobacteria, **TC** = total carbon, **TIC** = total inorganic carbon, **TOC** = total organic carbon, **WWTP** = wastewater treatment plant, **ZN** = Ziehl-Neelsen

Mycobacteria (family: Mycobacteriaceae) are widely distributed, not only in some parts of human and animal populations, but also in the environment (Kazda 2000; Kazda et al. 2009; Thoen et al. 2014; Varaine and Rich 2014). The genus *Mycobacterium* represents a group consisting of more than 180 presently known species and subspecies (LPSN 2016), which can be divided into two groups according to their occurrence (Kazda et al. 2009): (1) tuberculous mycobacteria (obligatory pathogenic mycobacteria) occur primarily in vertebrates and cause a severe disease known as tuberculosis. When introduced into the environment, obligatory pathogenic mycobacteria are capable of long-term (months or even years) survival but they are not able to proliferate. However, their survival is influenced by certain environmental conditions; i.e. in constant humidity they can survive in the absence of direct solar light and they can be transported by water and/or soil. (2) Non-tuberculous mycobacteria primarily inhabit the environment where they can also proliferate and degrade organic compounds. This mycobacterial group may occasionally infect vertebrates, including humans, and cause a disease known as mycobacteriosis. Members of the group occur in many different environments and metabolize various organic compounds. Under certain conditions (sufficient source of organic matter, permanent humidity etc.), these mycobacteria can also prevail in environments such as water sediments.

Underground systems in karstic regions reflect the status and progress on the karstic surface and closely communicate with it. Sooner or later, each inappropriate intervention into this surface ecosystem may increase the pollution of the cave with organic matter (Mohammadi and Shoja 2014). In the karstic underground, water from the water catchment area often travels over great distances and its path is sometimes difficult to trace. Karstic surface waters can be easily contaminated by chemical and/or microbiological pollutants which can be spread through the underground relatively quickly (Butscher et al. 2011). At the same time, these surface waters usually respond rapidly to flood waves, which run through the underground towards karst springs. It is therefore necessary to pay attention not only to the danger of all wastewater discharges but also to the appropriate management of agricultural areas (Andreo et al. 2006).

The Moravian Karst ("Moravsky kras") with its area of approximately 78 km^2 represents a unique region formed of unmetamorphosed limestone from middle and upper Devon. Water plays a crucial role in this area; allochthonous waters flowing from non-karstic areas enter into the underground almost directly at the geological boundary of the Devonian limestone (Hill and Polyak 2014; Terzic et al. 2014).

The high porosity of rocks creates a concentrated underground drainage. Underground spaces are basically collectors which concentrate water from karstic surfaces and which have largely the character of freely-flowing bodies of water with limited filtration and self-cleaning properties. Groundwater flows are generally characterised by lower retention capabilities and at the same time a relatively quick response to meteorological fluctuations (Parise and Lollino 2011). Due to these properties, they can be easily exposed to various pollutants, including bacteria (Zhang et al. 2014; Seman et al. 2015). However, a monograph describing the ecology of mycobacteria, which analysed the occurrence of mycobacteria in the environment and which was based on more than 6500 literary sources from databases including PubMed, Web of Science and others, did not mention any detection of mycobacteria in karstic environments (Kazda et al. 2009). Recent results showing the presence of mycobacteria in cave environments are also limited (Breitbart et al. 2009; Jurado et al. 2010; De Mandal et al. 2015a; De Mandal et al. 2015b). Due to the ubiquitous occurrence of mycobacteria in the environment it is supposed that they are transported to the cave system from the relevant water catchment area and that they are able to survive there in different matrices.

In light of the above, the first aim of this study was to evaluate the occurrence of mycobacteria in

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the ecosystem of the Moravian Karst based on the study of the environment in Bull Rock Cave ("Byci skala"), which is a part of the second longest cave system in the Moravian Karst. The second aim was to trace mycobacteria in the water catchment area of Jedovnice Brook flowing through this cave system and to assess the main sources of mycobacteria in the above-mentioned cave system. The third aim was to evaluate the geochemical characteristics of cave environment matrices and sediments of the water catchment area of Jedovnice Brook.

MATERIAL AND METHODS

Examination of the karstic cave system (Figure 1). Bull Rock Cave (approx. 7 km), together with Rudice Swallow Hole ("Rudicke propadani"; approx. 6 km) and Barova Cave ("Barova jeskyne"; approx. 0.9 km), is located in the central part of the Moravian Karst, the most extensive karstic area of the Czech Republic (Hromas 2009). This cave system is not accessible for the public, although a part of it is occasionally opened for visitors. This cave, with its length of almost 14 km, is a complex tunnel system partly with and partly without the active water flow of Jedovnice Brook. However, also the dry part of the cave system was swamped during the four cases of massive flooding in the past two centuries: 1832 (Jurende 1835), 1883 (Kriz 1891), 1927 (Prichystal and Naplava 1995) and 1972 (Burkhardt et al. 1972). Jedovnice Brook enters the cave system through the Rudice siphon (Hromas 2009). Barova and Bull Rock Caves are well-known archaeological and paleontological sites (Roblickova and Kana 2013). This cave system is partially located beneath agricultural as well as urbanised and forest landscape and it represents the largest locality of hibernating bats in the Czech Republic (Hromas 2009). The fauna, including non-vertebrates, of Bull Rock Cave was studied for the first time by Wankel (1856).

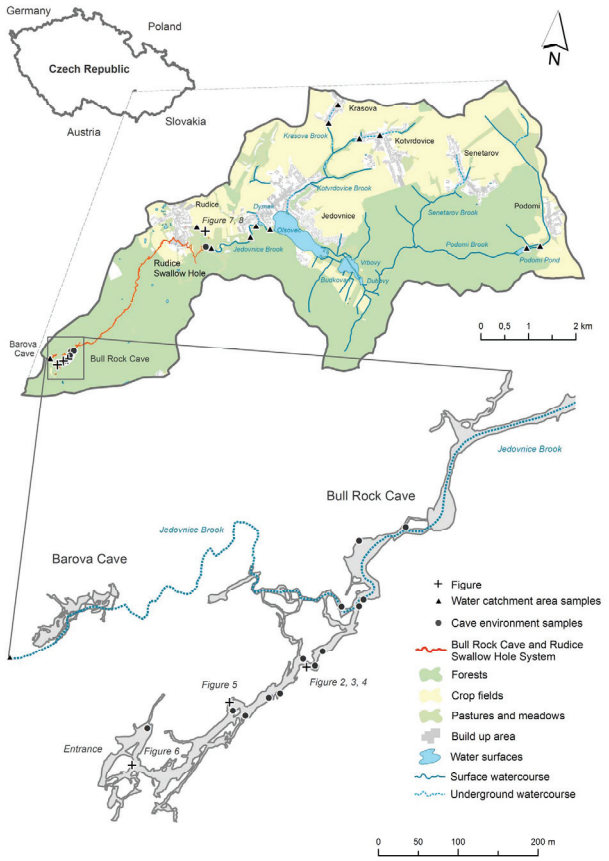

Figure 1. Water catchment area of Jedovnice Brook and Bull Rock Cave – Rudice Swallow Hole System

Examination and description of the water catchment area of Jedovnice Brook (Figure 1). Most of the water catchment area of Jedovnice Brook lies in the cadastral area of five municipalities (Habruvka, Jedovnice, Kotvrdovice, Rudice and Senetarov). Six villages (Jedovnice, Kotvrdovice, Krasova, Podomi, Rudice and Senetarov) are located in the studied area. The drainage basin covers an area of 70 km^2. Jedovnice Brook originates from four brooks (Krasova Brook, Kotvrdovice Brook, Senetarov Brook and Podomi Brook) which rise in agricultural landscape and flow through the villages with waste water treatment plants (WWTPs). These four brooks flow into the system of five Jedovnice ponds: Budkovan (7.07 ha), Dubovy (0.68 ha), Dymak (0.76 ha), Olsovec (42.00 ha) and Vrbovy (3.80 ha). The Olsovec Pond represents one of the major sources of organic matter brought into the cave system. Only 1.8 km beyond the outlet of Dymak Pond, Jedovnice brook goes below the ground in the Rudice Swallow Hole and springs to the surface again behind Bull Rock Cave and Barova Cave (length of the underground stream is 5.8 km). In the first part, the municipality of Rudice is located above the cave system, but in the other parts, Jedovnice Brook flows underground below the forest landscape. The northern part of the water catchment area is covered by crop fields, and arable land dominates the local landscape. While the municipality of Kotvrdovice manages its own simple settling tank, Krasova, Rudice and Senetarov's wastewater systems are connected to a new WWTP in Jedovnice.

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An evaluation of human activities influencing the studied area confirmed several types of risky behaviour connected with agriculture, production, residential and tourist functions of the territory. Although the intensity of the agricultural land use (35% of the area is agricultural land) is lower than in the rest of the Czech Republic (53% agricultural land), in this area the focus on crop production is more heavily emphasised, thus meaning that the proportion of arable land is higher in the monitored area compared to the rest of the country (Spatial analytical materials published by the Czech Statistical Office; CSO 2016a).

Three big farms situated in Jedovnice and Senetarov dominate the agriculture of the region and they also rear livestock (cattle, sheep, horses etc.) in the pastures, which is less common in the studied region than in the rest of the Czech Republic (List of subsidy beneficiaries monitored by the State Agricultural Interventional Fund; SAIF 2016). Manure was stored in the fields and used for fertilisation. The use of cattle manure as a supplement for feeding carps was documented at least once in the ponds on Jedovnice Brook (unpublished observations).

Forests cover more than half of the water catchment area; the coefficient of ecological stability of the territory is therefore high. Forests, karst elements and ponds in the areas attract many tourists, who are concentrated especially in Jedovnice. Every year, more than 15 thousand tourists stay on average for two days in this municipality, in a campsite or in one of the 12 accommodation facilities (Spatial analytical materials and Public Database published by the Czech Statistical Office; CSO 2016b). Olsovec Pond is intensively used for leisure activities as well as for fish husbandry (the remaining four ponds, Dubovy, Budkovan, Vrbovy and Dymak, are used for fish husbandry only).

The area also contains a relatively large number of human settlements. However, the municipalities located in the water catchment area are rather small. Two of the six villages have less than five hundred inhabitants (Krasova and Podomi), between 501 and 1000 people live in three of the villages (Kotvrdovice, Rudice and Senetarov), and 2700 inhabitants live in Jedovnice. Household sewage pollution is relatively low since all of the local settlements are connected to wastewater treatment plants. Nevertheless, some houses and cottage settlements are still not connected to sewerage (Population and Housing Census 2011 published by the Czech Statistical Office; CSO 2016c).

Origin of the samples. A total of 68 samples for mycobacteria detection were collected in the cave environment (50 samples) and in the water catchment area of Jedovnice Brook (18 samples). Sediments, faeces and other solid samples were taken by sterile tongue depressor and put into sterile plastic bags for culture examination and into sterile Eppendorf tubes for DNA isolation. Stalactite scrapings with drip water were collected by a sterile tampon into sterile disposable plastic containers (30 ml) and into sterile Eppendorf tubes. After collection, the samples in plastic bags or containers were kept at +6 °C for up to one week before bacteriological analysis; DNA from samples in Eppendorf tubes was either isolated immediately or else the samples were frozen at –20 °C. All these samples were examined for the presence of mycobacteria by direct microscopy examination after Ziehl-Neelsen (ZN) staining, culture, and qPCR for direct detection of mycobacterial DNA in the matrix (Table 1). A total of 26 samples were analysed by geochemical methods: 14 samples were collected from the Bull Rock Cave environment and 12 samples were collected from the water catchment area of Jedovnice Brook, including the karst spring behind Bull Rock Cave (Figure 1, Table 2).

Cave system. A total of 50 samples for mycobacteria detection which were collected from an indoor environment (both parts with and without active water flow of Jedovnice Brook) of Bull Rock Cave were divided into three types (Table 1): (1) drip water and brook sediments (23 samples; Figure 2); (2) alluvial plant materials, consisting of wood fragments and other plant material from trees, grass etc. (12 samples; Figure 3) and (3) earthworm castings (Figure 4) and bat guano (15 samples; Figures 5 and 6).

Water catchment area. The 18 samples for mycobacteria detection consisted of brook sediments collected in the appointed localities (Table 1): nine samples in the village upstream of the WWTP (Figure 7) and nine samples from the outflow from the WWTP (Figure 8).

Microscopic examination. Before culture, samples were stained according to ZN and examined by light microscopy for the presence of acid-fast rods (AFR). At least 200 fields of view were examined in each sample (Ulmann et al. 2015).

Culture examination. Liquid matrices (30 ml) were sampled in sterile plastic containers and

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Table 1. Mycobacteria detection by ZN[a] staining, culture examination and direct DNA isolation from 68 environmental samples from the Bull Rock Cave and the water catchment area of Jedovnice Brook

Sample origin	Matrix examined	No.	ZN[a] and/or culture			DNA detection[b]	
			result	No.	%	No.	%
Cave	Drip water and brook sediments	23	–	15		7/15	46.7
			+	8	34.8	7/8	87.5
						14/23	60.9
	Alluvial plant material	12	–	8		7/8	87.5
			+	4	33.3	2/4	50.0
						9/12	75.0
	Earthworm castings and bat guano[c]	15	–	4		4/4	100
			+	11	73.3	10/11	90.9
						14/15	93.3
Subtotal		50	–	27		18/27	66.7
			+	23	46.0	19/23	82.6
						37/50	74.0
Water catchment area	Sediments from the villages upstream of the WWTPs	9	–	7		4/7	57.1
			+	2	22.2	1/2	50.0
						5/9	55.5
	Sediments downstream of the WWTP outflow	9	–	2		1/2	50.0
			+	7	77.8	4/7	57.1
						5/9	55.5
Subtotal		18	–	9		5/9	55.6
			+	9	50.0	5/9	55.6
						10/18	55.6
Total		68	–	36		23/36	63.9
			+	32	47.1	24/32	75.0
						47/68	69.1

WWTP = waste water treatment plant

[a]Ziehl-Neelsen staining

[b]Mycobacterial DNA was confirmed by different methods directly in the matrix of the sample

[c]Three samples of bat guano were positive in all examinations

centrifuged at 3200 × *g* for 20 min. Hard matrices (30 g), i.e. sediments, were treated by adding 30 ml distilled water and after 10 min of extensive shaking they were let to settle for an additional 5 min. A total of 10 ml of turbid supernatant was transferred to a new centrifuge container and cen-

Table 2. Geochemical characteristics of collected samples (n = 26; mean ± SEM)

Sample origin	No.	Parameters				
		pH	conductivity (µS/cm)	TOC (%)	TIC (%)	LOI (%)
Cave environment						
Drip water and brook sediments	2	8.01 ± 0.02	82.45 ± 7.75	0.05 ± 0.00[a]	–	2.54 ± 0.66[a]
Alluvial plant materials	4	6.65 ± 0.60	373.13 ± 164.16	24.85 ± 6.54[b]	1.90 ± 1.12	–
Earthworm castings	8	6.86 ± 0.16	292.01 ± 93.82	1.68 ± 0.37[a]	0.30 ± 0.06	6.24 ± 1.00[ab]
Water catchment area of Jedovnice Brook						
Sediments from the villages upstream of the WWTPs	6	6.63 ± 0.17	183.33 ± 57.04	1.34 ± 0.36[a]	0.41 ± 0.17	6.21 ± 1.71[ab]
Sediments downstream of the WWTP outflow	6	6.62 ± 0.16	441.68 ± 199.59	2.51 ± 0.42[a]	0.65 ± 0.18	10.79 ± 1.96[b]

LOI = loss on ignition; TIC = total inorganic carbon; TOC = total organic carbon; WWTP = wastewater treatment plant

[a,b]Significant differences between groups (P < 0.05)

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Figure 2. Drip water sediment close to the Map of the Republic ("Mapa Republiky" sinter formation); drip water is collected in a pot for consumption before and after cooking. Volume of drip water is ca. 0.01–1.0% from the total water flow (Photo I. Pavlik)

trifuged for 20 min at 3200 × *g*. Approximately 5 g of alluvial plant or other material were, according to its consistency, grinded in a mortar or extensively vortexed with glass beads for 10–30 min until maximal homogeneity was achieved; then 5 ml of sterile saline were added, the suspension was mixed and filtered through sterile gauze. Pellets and filtrates were homogenised and decontaminated with 4% NaOH and lauryl-sulphate according to the methods described previously by Engbaek et al. (1967) and Brooks et al. (1984). A total of 800 µl of suspensions were inoculated by 200 µl in duplicate into two slants with Lowenstein-Jensen (L-J) medium without and two L-J mediums with sodium-pyruvate. Incubations were performed in parallel for three months, with one L-J medium type, at 28 °C and 37 °C. Mycobacterial growth was examined after the first week and then every second week (Ulmann et al. 2015).

Figure 3. Wooden material consisted of alluvial wood (two branches around 20 cm long) and alluvial leaf (one brown decaying leaf on the right side), in Jedovnice River sediment behind the Schenk Siphon; rapid stream flow from Jedovnice Brook is on the left (Photo I. Pavlik)

Figure 4. Fresh earthworm castings after a few days of flooding behind the Schenk Siphon, close to the underground course of Jedovnice Brook (Photo I. Pavlik, July 14, 2016)

Figure 5. In some parts of the cave the number of overwintering bats is relatively high (Photo I. Pavlik)

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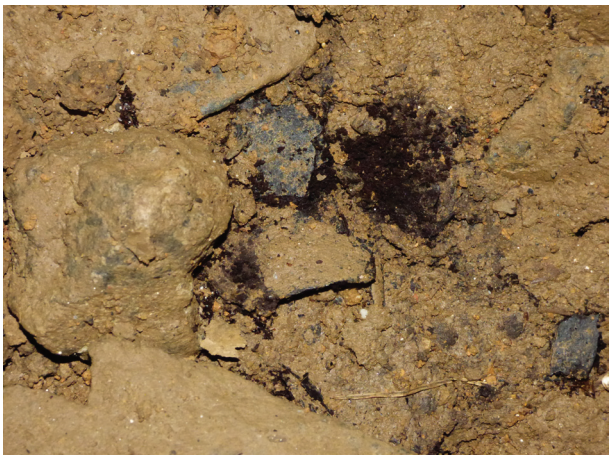

Figure 6. In some parts of the cave decaying bat guano is concentrated on the ground under the bats (Photo I. Pavlik)

Identification of mycobacterial isolates and mycobacterial DNA detection in samples. The first examination of cultivated mycobacterial strains was carried out by macroscopic and microscopic examination after ZN staining. For DNA analysis samples were frozen at –20 °C or used immediately for DNA analysis. The DNA extraction was performed using a commercial kit (QIAGEN QIAamp DNA Stool Mini Kit). Preliminary identification was made by GenoType® Mycobacterium CM/AS (Hain Lifescience GmbH, Nehren, Germany); mixed and slightly contaminated cultures were re-isolated and isolates were subjected to further identification. All DNA was examined using the PCR method for the detection of the 16S rRNA gene to check the quality of the DNA. Universal bacterial primers 5'-CCT ACG GGN GGC WGC AG-3' and 5'-GAC TAC HVG GGT ATC TAA TCC-3' of the V3 and V4 variable regions were used (Nossa et al. 2010). The PCR reaction mixture was prepared using MAXIMA Probe/ROX qPCR MasterMix (Thermo Fisher Scientific) in a total volume of 20 µl. The PCR program (PTC-200, MJResearch thermocycler) included a denaturation step of 10 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 120 s at 72 °C, and a final elongation step of 5 min at 72 °C. Amplification with universal primers was carried out to verify that DNA extraction was successful and that no inhibitors would prevent the mycobacteria-specific amplification process. Additionally, in the samples which were negative for the 16S rRNA PCR reaction, the commercial "*Mycobacterium tuberculosis* DNA Detection Kit" provided by the company genetrac, Ltd. (Czech Republic) was used for the control of PCR inhibition using the "Internal standard" present in the MasterMix (http://www.genetrac.com/en/mycobacterium).

Figure 7. Sewage disposal plant located in the lower part of the village Rudice was closed in April 2016 (since then the wastewater is treated in the wastewater treatment plant in Jedovnice village; Photo I. Pavlik)

Figure 8. Grey sewage (grey wastewater) was running to the Rudice Swallow Hole until the closure the Rudice Sewage disposal plant (Photo I. Pavlik)

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The *Mycobacterium* genus-specific primers 5'-ACC AAC GAT GGT GTG TCC AT-3' and 5'-CTT GTC GAA CCG CAT ACC CT-3' were used for DNA amplification of the hsp65 gene (Telenti et al. 1993); the specific primers 5'-GTS CAR AAC GAR ATC GAR CG-3' and 5'-CCB GAY TCR CCC CAG ATG AA-3' were used for DNA amplification of *dnaA* gene (Mukai et al. 2006). The PCR reaction mixtures for both hsp65 and *dnaA* systems were prepared using the MAXIMA Probe/ROX qPCR MasterMix (Thermo Fisher Scientific) in a total volume of 20 µl. The PCR program included a denaturation step of 10 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C, 60 s at 72 °C, and a final elongation step of 2 min at 72 °C.

The resulting amplicons obtained by both of the methods were visualized by UV illumination of an agarose gel, followed by purification using the QIAGEN QIAquick Gel Extraction Kit and were sequenced at GATC Biotech (Konstanz, Germany). Identification of the species that most closely matched the sequence results for the target gene was done using BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EZ Taxon searching (http://ezbiocloud.net/).

M. avium complex members were identified in the specific qPCR reactions for the detection of the following insertion sequences: IS*901* specific for *M. avium* subsp. *avium* and IS*1245* specific for *M. avium* subsp. *hominissuis*. This qPCR mixture was prepared using the MAXIMA Probe/ROX qPCR MasterMix (Thermo Fisher Scientific) in a total volume of 20 µl. The qPCR conditions used were exactly as described in (Slana et al. 2010).

Geochemical characteristics of the examined samples. The measured geochemical characteristics of the samples were pH, conductivity, total organic carbon (TOC), total inorganic carbon (TIC) and loss on ignition (LOI). A total of 26 samples were analysed: 14 samples from cave environments (two drip water and brook sediments, four alluvial plant materials and eight earthworm casts) and 12 samples from the water catchment area of Jedovnice Brook (six from the villages upstream of the WWTP and six from the outflow of the WWTP). The samples were analysed immediately after the sampling.

pH. A soil active reaction (pH/H_2O) was performed in all of the samples according to the ISO 10390 Method (Soil quality – determination of pH). Solid matrices underwent an infusion by distilled water and resulting suspensions were shaken and prepared for measuring by the WTW InoLab Multi 720 tool with SenTix 41 electrode.

Conductivity. The conductivity measurements were performed according to ISO 11265:1994 – Soil quality – Determination of the specific electrical conductivity. Sample aliquots were mixed with distilled water (1 : 5) and were shaken for 30 min. A multimeter WTW Multi 3320 with TetraCon 325 electrode was used for measurements.

Total organic carbon and total inorganic carbon. Total organic and inorganic carbon were analysed using the Analytik Jena multi N/C 2100S with HT 1300 module according to the standard ISO 10694 Method (Soil quality – Determination of organic and total carbon after dry combustion; elementary analysis). Calcium carbonate and tetrasodium ethylenediamine tetraacetate-tetra-hydrate were used for calibrations. A homogenised sample (all samples were subjected to homogenization using 40–50 g of dry sample) was weighed and carried into the furnace. This was burned at 1400 °C and the final CO_2 concentration was reported as a peak in direct proportion to total carbon (TC). The same sample was rid of carbonates which were decomposed using hydrochloric acid in order to allow analysis. The analysis of TOC was performed in the same manner. The following equation was used for TIC measurement:

$$TIC = TC - TOC$$

where: TIC = total inorganic carbon; TC = total carbon; TOC = total organic carbon

Water content analysis. The samples were dried to a constant weight in 105 °C in a drying box. Water content was calculated on the basis of difference between non-dried sample and dried sample.

Loss on ignition. A homogenised sample was annealed at a temperature of 550 ± 25 °C in a furnace to constant weight. The analysis was performed according to a technical standard. The following characteristics of waste were used: determination of loss on ignition in waste, sludge and sediments (ISO/TR 18230:2015).

Statistical analysis of the results. The two-tailed Fisher's exact test, which is a part of the GraphPad Prism v5.02 programme (GraphPad Software, Inc., USA), was performed to determine whether there were significant differences between different pairs

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of sample types in mycobacteria detection. The results of the geochemical parameters were analysed using the statistical package Unistat 6.1. (Unistat Ltd., Great Britain). For all variables tested, normality and homogeneity of variances were checked by means of a Shapiro-Wilk test. The data were subjected to a one-way ANOVA and subsequently to a Tukey-HSD test for multiple comparisons in order to assess the statistical significance of the differences among all possible pairs of groups. The differences were considered significant at $P < 0.05$.

RESULTS

Mycobacteria detection in examined matrices (Table 1)

In total, mycobacteria were detected by ZN staining and culture examination in 47.1% of the examined samples (32 positive out of the 68 samples; 32/68). Mycobacteria detection was higher in the water catchment area of Jedovnice Brook (50.0%, 9/18) in comparison to the cave environment (46.0%, 23/50; $P < 0.05$). In the cave environment, the earthworm castings and bat guano were statistically more likely to be positive for mycobacteria (73.3%, 11/15) in comparison to drip water and brook sediments (34.8%, 8/23; $P < 0.0001$) and alluvial plant material (33.3%, 4/12; $P < 0.01$). In the water catchment area of Jedovnice Brook a higher positivity (77.8%, 7/9) was detected in the brook sediments collected downstream from the WWTP outflow in comparison to the brook sediments (22.2%, 2/9) from the villages upstream of the WWTP.

Mycobacterial DNA was detected in 69.1% (47/68) of the samples (no inhibition was found; negative samples were positive for amplification with the V3/V4universal primers). The detection rate was independent of ZN and/or culture positivity: in ZN and/or culture-negative samples ($n = 36$), mycobacterial DNA was found in 23 (63.9%) samples and in ZN and/or culture-positive samples ($n = 32$) the positivity for mycobacterial DNA was 75.0% ($n = 24$).

In the cave environment, mycobacterial DNA was detected in 74.0% (37/50) of the examined samples. The highest positivity was documented in earthworm castings and bat guano (93.3%, 14/15), followed by alluvial plant material (75.0%, 9/12) and drip water and brook sediments (60.9%, 14/23). In the water catchment area of Jedovnice Brook mycobacterial DNA was detected in 55.6% (10/18) of the samples. The same mycobacterial DNA positivity was found in the brook sediments from the village upstream of the WWTP and from the WWTP outflow.

Mycobacterial species present in the examined samples (Table 3)

In the water catchment area of Jedovnice Brook eleven mycobacterial species (*M. xenopi*, *M. goodii*, *M. chelonae*, *M. rhodesiae*, *M. insubricum*, *M. terrae*, *M. fortuitum*, *M. parascrofulaceum*, *M. avium* subsp. *hominissuis*, *M. peregrinum*, and *M. porcinum*) were detected. *M. xenopi*, *M. chelonae*, *M. rhodesiae* and *M. goodii* were found only in the surface environment samples from the water sediments from the water catchment area of Jedovnice Brook, whereas *M. goodie* and *M. chelonae* were only found upstream of the WWTPs, and *M. rhodesiae* only downstream of the WWTPs.

Species homogeneity/heterogeneity in examined matrices and isolates

Out of the 59 positive samples from the cave environment 23 (39.0%) belonged to slowly growing and 36 (61.0%) to rapidly growing mycobacteria. Only one species (*M. terrae*) was detected in all of the examined types of the matrices and isolates, both from the cave environment and from the water catchment area of Jedovnice Brook. *M. avium* complex members and *M. parascrofulaceum*, *M. fortuitum*, *M. porcinum*, *M. peregrinum* and *M. insubricum* were identified both in the cave environment (minimally one type of matrix) and in the water catchment area of Jedovnice Brook. Faeces from earthworms and bats displayed the highest degree of species heterogeneity (23 hits; nine slowly and 14 rapidly growing mycobacteria), followed by alluvial plant material (19 hits; five slowly and 14 rapidly growing mycobacteria) and water sediments (17 hits; nine slowly and eight rapidly growing mycobacteria).

Cave environment

A total of eight slowly growing mycobacteria (*M. terrae*, *M. arupense*, *M. gordonae*, *M. lentiflavum*, *M. parascrofulaceum*, *M. parmense*, *M.*

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Table 3. Number of PCR-positive results for individual mycobacterial species detected in examined samples

	Water sediment	Alluvial material	Faeces	WWTP	
				U	D
M. xenopi (3)*	0	0	0	2	1
M. goodii (2)	0	0	0	1	1
M. chelonae (1)*	0	0	0	1	0
M. rhodesiae (1)	0	0	0	0	1
M. insubricum (14)	5	0	5	2	2
M. terrae (11)*	3	1	4	1	2
M. fortuitum (9)*	0	4	3	1	1
M. parascrofulaceum (5)	0	1	1	3	0
M. a. hominissuis (4)*	1	1	1	1	0
M. peregrinum (3)	0	0	1	0	2
M. porcinum (2)	0	0	1	1	0
M. mageritense (6)	1	1	4	0	0
M. parmense (3)	2	0	1	0	0
M. arupense (2)*	2	0	0	0	0
M. gordonae (1)	0	0	1	0	0
M. saskatchewanense (1)	0	0	1	0	0
M. a. avium (1)*	1	0	0	0	0
M. poriferae (1)	1	0	0	0	0
M. lentiflavum (1)*	0	1	0	0	0
M. simiae (1)	0	1	0	0	0
M. chubuense (1)	0	1	0	0	0
M. flavescens (1)	0	1	0	0	0
M. gilvum (1)	0	1	0	0	0
M. vanbaalenii (1)	0	1	0	0	0
M. gadium (1)	0	1	0	0	0
M. species (5)	1	4	0	0	0
Total (82)	17	19	23	13	10

D = downstream, *M. a. avium* = *M. avium* subsp. *avium*; *M. a. hominissuis* = *M. avium* subsp. *hominissuis*, U = upstream, WWTP = waste water treatment plant

*Clinically important/relevant mycobacterial species

saskatchewanense and *M. simiae*), two members of the *M. avium* complex (*M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*) and members of the *M. terrae* and *M. avium* complexes were detected. Only one species (*M. terrae*; six hits) was detected in all three types of examined matrix, followed by *M. avium* (four hits: *M. avium* complex – two hits and *M. avium* subsp. *avium* – one hit and *M. avium* subsp. *hominissuis* – one hit) and *M. parmense* (three hits). Higher species heterogeneity was found among the rapidly growing mycobacteria. *M. insubricum* (10 hits), the most common species in the cave environment, was detected in faeces from earthworms and bats (five hits) and in water sediments (five hits). The second most common species were *M. fortuitum* (six hits) and *M. mageritense* (six hits). Only *M. mageritense* was documented in all three matrices; the highest frequency was found in faeces of earthworms and bats (four hits). The lowest heterogeneity was found in the water sediments (only two rapidly growing species) in comparison to alluvial material (seven species) and faeces of earthworms and bats (six species).

Geochemical characteristics of the examined samples (Table 2)

In the cave environment, a deposited cone had the lowest pH level (5.03) and water sediments had the highest pH level (8.01). The highest content of organic carbon was found in plant alluvial material, whereas the LOI was below the limit of detection in these samples. The average level of TOC in plant alluvial material was significantly higher ($P < 0.001$) compared to all other types of samples. A statistically significant difference ($P < 0.05$) was found in the LOI level between water sediments in the cave environment and water sediments from the outflow of the WWTPs.

DISCUSSION

The difficulty in detecting mycobacteria using culture examination in the environment might be one reason for the current low interest in the presence or absence of mycobacteria in karstic cave systems. The presence of contaminating sporogenic bacteria and moulds that destroy the culture media within a few hours or days represent the main issue. Rapidly growing mycobacterial species require incubation times of up to one week and slowly growing mycobacterial species require even weeks or months (Wayne and Kubica 1986). In our study this contamination was characteristic especially of sediments from the surface close to the WWTP outflow which is the reason for lower mycobacterial detection by culture examination in comparison with PCR (Table 1). WWTPs serve as a rich environment for a large spectrum of microbial communities (Hu et al. 2012) which made it impossible to grow mycobacteria in culture media

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without sufficient prior decontamination measures. The temperature, media composition, etc., required for successful isolation *in vitro* is also not exactly known for these environmental mycobacteria (Kazda et al. 2009). The highest detection rate of mycobacterial DNA shown in Table 1 could be caused by the detection of dead or living but not *in vitro* culturable environmental mycobacteria.

Data on the detection of mycobacteria in caves is quite limited (Breitbart et al. 2009; Jurado et al. 2010; De Mandal et al. 2015a; De Mandal et al. 2015b). However, there are previously published studies describing the massive occurrence of different species of moulds in Moravian and Slovak karstic caves (Lukesova and Novakova 2009; Novakova 2009; Hubka et al. 2015). The presence of moulds could be an important stimulating factor for different bacterial species (Novakova 2009), including mycobacteria (Kazda et al. 2009).

The decaying organic material in the Moravian Karst caves was first observed centuries ago (Hertod-Todtenfeldt 1669; Nagel 1749; Mayer 1781; Wankel 1882). The strongly-smelling alluvial sediments in these caves contain pieces of wood, foliage, and pine-needles mixed together with sand, and was studied by Kriz (1891; 1892). The impact of alluvial sediments on geomicrobiology was investigated recently by Shabarova et al. (2014). This study verified the hypothesis that the long-term residence of water in stagnant cave pools consistently led to a decline in bacterial diversity. The dynamics of different prokaryotes, viruses, and heterotrophic nanoflagellates in karstic regions were described also by Wilhartitz et al. (2013). The documented changes that occur under these conditions could give an important competitive advantage to some bacteria, such as those of the genus *Mycobacterium*.

The cave environment in karstic areas is impacted by human activities both directly and indirectly. Caving activity, access to the caves, the numbers of people visiting the cave, etc., are examples of direct influences. Indirect influences include agriculture, transportation, mining and pollution of the surrounding surface (Faimon et al. 2006; Einsiedl et al. 2010). The attractive infrastructure that has been developed supports the tourist function of the studied area in the Moravian Karst (Migon 2011). Contemporary lifestyles, the current density of settlements and the relatively high levels of tourists in some parts of the Moravian Karst (unpublished observation) lead to a number of problems in terms of both the surface and underground protection. The above-mentioned factors could explain the high presence of cultured mycobacteria in the water sediments collected from the outflow from the WWTPs in the villages. Previous studies found no differences in mycobacterial occurrence between treated and non-treated sewage water, which demonstrates the risk of cave system contamination by surface stream water (Schuppler et al. 1995; Korzeniewska 2011). We made the same observations (PCR-positive results) in the water catchment area (Table 1).

The 65-kDa heat shock protein gene (*hsp65*) has been used for more than two decades to identify and detect *M.* species. Originally, a 441 bp region of the gene was used by Telenti et al. (1993) for restriction analysis. One of the earliest studies utilising *hsp65* gene sequencing for mycobacterial identification was carried out by Kapur et al. (1995). This method, based on the 441 bp region, was later evaluated and tested on several hundreds of smear-positive sputa samples, type strains, routine identification of mycobacterial isolates from clinical specimens, and species identification of mycobacteria present in primary liquid detection media (McNabb et al. 2006). Comparison of *hsp65* sequencing with the Accu-Probe (GenProbe) indicated that sequencing allowed for identification of probe-negative species (McNabb et al. 2004).

The identification of mycobacteria by *hsp65* sequencing is limited by the lack of valid sequences in data repositories. In the GenBank database a total of 5344 sequences of mycobacterial *hsp65* are available (July 17, 2016), out of which 4597 belong to *M. tuberculosis*. The rest of the sequences belong to a further 21 mycobacterial species. Another problem of this method is the similarity of many sequences belonging to other bacterial species (*Nocardia*, *Streptomyces*, etc.) which can be found in the database. McNabb et al. (2004) reported that for nearly 15% of the clinical isolates for which *hsp65* analysis was attempted, analysis was hampered by the lack of sequence information in internet repositories. Additionally, *hsp65* sequencing cannot distinguish between members of the *M. tuberculosis* complex (with the exception of *M. canettii*) and the members of the *M. avium* complex. A similar study was conducted by van der Wielen et al. (2013) in drinking water; *M. avium*, *M. genavense*, and *M. gordonae* were identified by *hsp*65 gene sequencing. *M. avium* and *M. gordonae* were detected in our study (Table 3).

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In light of the above, samples which were identified as *M. avium* complex members were tested for the presence of specific sequences. Although Turenne et al. (2006) recommended a specific part of the *hsp65* gene for species differentiation of this complex system, it has not been commonly used. Instead, a method based on identification of the insertion sequence IS*901* (specific for *M. avium* subsp. *avium*) and IS*1245* (specific for *M. avium* subsp. *hominissuis*) by PCR is commonly used as a standard method of differentiation of *M. avium* complex members (Moravkova et al. 2008). A triplex qPCR reaction (Slana et al. 2010), which is based on the simultaneous detection of specific insertion sequences, and which also contains an internal amplification control enabling identification of false-negative results, was used in our study.

One isolate identified as *M. avium* subsp. *avium* which was detected in the water sediments in a cave could have been introduced to this environment with wastewater polluted by domestic hen faeces, as hens were extensively reared in all villages in the water catchment area of the present study (unpublished observations). Also, one *M. avium* subsp. *hominissuis* isolate originated from all types of samples in the cave environment (Table 3). This finding is not surprising because this member of the *M. avium* complex is a common part of sawdust, bark and other decaying parts of plant tissues and wooden material (Kazda et al. 2009).

Blackwood et al. (2000) and Mukai et al. (2006) used chromosomal replication initiation protein as a target for detection of environmental species such as *M. terrae*, *M. fortuitum* and *M. parafortuitum*. The gene is also suitable for differentiation of *M. tuberculosis* complex strains (Mukai et al. 2006). Some authors have recommended *dnaA* gene sequencing as a complementary method to 16S rRNA gene sequencing (Blackwood et al. 2000). However, the problem with valid sequences in data repositories is similar as described for *hsp65* above. In the GenBank database a total of 22 351 sequences of mycobacterial *dnaA* are available. Most of the sequences are specific for *M. tuberculosis* strains (20 667). The rest of the sequences belong only to 34 other mycobacterial species. Therefore, the identification of mycobacterial species is also limited by the insufficient amount of sequencing data available.

Other frequently used targets for differentiation of closely related mycobacterial species are the β-subunit of the bacterial DNA-dependent RNA polymerase gene (*rpoB*), the 16S rRNA gene or the 16S-23S ITS region (Persing 2011). The 16S rRNA gene is universally present in all bacterial species (Weisburg et al. 1991; Coenye and Vandamme 2003; Tang et al. 2014), so we used it as a control of the quality of the isolated DNA for PCR. The DNA isolates with negative PCR results were isolated again. The DNA isolates with positive PCR results were used for amplification of the *hsp65* or *dnaA* genes and sequencing. Sequencing data for both *hsp65* and *dnaA* were evaluated, compared and used to define the mycobacterial species as listed in Tables 1 and 3.

The highest detection rate in the cave environment was found in the earthworm castings and bat guano, which reached up to 73.3% (11/15; Table 1). Over the years, research in Bull Rock Cave has resulted in the identification of the following terrestrial earthworm species (Cernosvitov 1935; Stastna et al. 2003; Pizl et al. 2008): *Allolobophora chlorotica* (Savigni 1826), *Dendrobaena octaedra* (Savigni 1826), *Dendrobaena rubidus* (Savigni 1826), *Eiseniella tetraedra* (Savigni 1826), *Proctodrilus tuberculatus* (Cernosvitov 1935), *Stylodrilus brachystylus* (Hrabe 1929) and *Trichodrilus moravicus* (Hrabe 1938). In fresh alluvial sediments in Bull Rock Cave between 200 and 300 earthworms per 1 m^2 were observed (Karel Kovarik, personal communication). The impact of terrestrial earthworms on the spread of mycobacteria was described by Fischer et al. (2003); this finding could be relevant for the detection of mycobacteria in earthworm castings in the cave environment. The earthworm castings and the geochemical parameters of the sediment (Table 2) in the cave environment provided optimal conditions for the survival of mycobacteria.

Bull Rock Cave is one of the most important caves for the overwintering of bats in the Moravian Karst and in the wider geographical region. In 2010, a total of 1887 bats of eight species: *Myotis myotis* (1254 animals), *Myotis emarginatus* (57 animals), *Myotis nattereri* (eight animals), *Myotis daubentonii* (16 animals), *Myotis dasycneme* (four animals), *Pipistrellus pipistrellus* (30 animals), *Barbastella barbastellus* (13 animals) and *Rhinolophus hipposideros* (505 animals) were counted (Vlastislav Kana, unpublished data). This means that both of these organisms (earthworms and bats) could spread the mycobacteria through the Bull Rock Cave environment as was described in bat guano in caves in India (De Mandal et al. 2015a; De Mandal et al. 2015b).

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Compared to other types of samples in the cave environment, the lowest positivity of mycobacterial detection was in drip water and brook sediments (60.9%, 14/23; Table 1), which can be related to the lowest concentration of organic carbon in these matrices. A higher concentration of organic carbon, however, did not influence mycobacterial prevalence in alluvial plant material. Drip water and brook sediments from the cave environment were slightly alkaline, with average pH values of around eight. These differences were not significant compared to other types of samples which had neutral pH values because of the small amount of water sediments in the cave environment. A significantly higher concentration of LOI in sediments downstream of the WWTPs indicates a higher content of non-volatile organic compounds although TOC increased only slightly (Table 2).

The water catchment area of Jedovnice Brook is burdened by household wastewater and by the tourist activity in the area, i.e. the number of tourists. The tourist areas (in particular, cottage settlements) and some houses (especially in the village Kotvrdovice) are still not connected to the WWTPs (unpublished observation). This wastewater flows as a part of Jedovnice Brook into the studied cave systems (Figure 1). Its smell was documented in the cave environment multiple times during the studied period, especially after a strong rain (unpublished observation). Considering that mycobacteria were detected in different types of waste, including communal waste (Kazda et al. 2009), it could be expected that the wastewater (including treated wastewater from the WWTP in Jedovnice) represented the most significant source of mycobacterial contamination (Figures 7 and 8).

Even though the intensity of agricultural land use is lower than in the rest of the Czech Republic, the local agriculture focuses on crop production, resulting in the elution of fertilizers into the surface water of Jedovnice Brook (Figure 1). The expected increase in pastures and meadows instead of arable land in protected areas could decrease this risk.

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