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Spacio-Temporal Distribution and Tourist Impact on Airborne Bacteria in a Cave (Škocjan Caves, Slovenia)

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Abstract: (1) Background: Airborne microbes are an integral part of a cave ecosystem. Cave allochtonous airborne microbiota, which occurs mainly during aerosolization from an underground river, from animals, and from visitors, is particularly pronounced in show caves. The impacts of tourists and natural river aerosolization on the cave air were estimated in large cave spaces within the Škocjan Caves; (2) Methods: Simultaneously with the measurements of atmospheric parameters, cultivable airborne bacteria were impacted, counted and identified using MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry); (3) Results: A mix of bacteria typically associated with humans and with natural habitats, including a large percentage of non-identified isolates, was found in the cave air. Few of the isolates were attributed to Risk Group 2. A strong positive correlation between tourist numbers and the rise in the concentration of airborne bacteria was indicated. Concentration of airborne bacteria rises to particularly high levels close to the underground river during periods of high discharge. A 10-times lower discharge reflected an approximately 20-times lower concentration of airborne bacteria; (4) Conclusions: Caves that are open and visited contain a diverse airborne microbiota originating from different sources. Enormous cave chambers that display relatively dynamic cave climate conditions do not normally support the enhancement of airborne bacterial concentrations.

Keywords: show cave; tourism impact; aerobiology; biomass; MALDI-TOF MS

1. Introduction

Some microorganisms have their own ability to move which, on the micro scale, serves to support active movement, searching for nutrients and new ecological niches [1]. For long-distance transport, microbes and other minute particles use one of three modes of dispersion: air (anemochoric), water (hydrochloric), or biological vectors (biochoric), the latter via either animal or human vectors [2]. When airborne, microbes can travel reasonably great distances and can have a global impact on ecosystems [3]. Results of laboratory experiments provide fundamental data on the transport and fate of fine particles, but in some cases it is appropriate to adopt a natural model system.

An example of natural, more or less confined, systems is provided by caves, which are advantageous for some physiological and ecological studies because they lack various environmental

stressors, such as UV irradiation and desiccation. On the other hand, elevated concentrations of radon and its decay products might have a significant impact on airborne microbiota [4]. Some microorganisms are already airborne when they enter caves from the external surface, whereas others become airborne due to the splashing of water or the presence and activity of animals and humans. This latter effect is best observed in show caves, where visitors introduce and spread many inanimate and living particles [5–8]. An example from an indoor environment, a children's classroom, showed that the emission rates of microbes from human skin can range from 0.8 to 35 $\times$ 10^6 bacterial cells per person-hour and from 3 to 57 $\times$ 10^6 fungal cells per person-hour [9]. Dissemination of detached skin scales is not their only influence because humans impact the air quality directly, by breathing, coughing, and sneezing. Related droplets and aerosols contain numerous microorganisms originating from the upper respiratory tract and from the oral mucosa, and these are part of the normal, persistent, or transient microbiota. Clothes and lint can also serve as a source for airborne bacteria and fungi. Once they are incorporated within an aerosol, microorganisms persist in the air for relatively long periods, and some of them have the potential to cause infections in susceptible hosts. Frequently-encountered bacterial genera are *Staphylococcus*, *Bacillus*, *Kocuria*, *Micrococcus*, *Corynebacterium*, and *Arthrobacter*, alongside fungi from genera, such as *Candida*, *Aureobasidium*, *Penicillium*, and *Phoma* [10], and many other microorganisms were revealed using culture-independent techniques [11]. These microbes can remain viable on various surfaces for periods ranging from days to months, depending upon the species and the environmental conditions, particularly the temperature and relative humidity [12].

The airborne microbiota, together with microenvironmental and climatic disturbances caused by human activities [4,8,13,14], can contribute to biodeterioration of many susceptible items preserved underground, especially artefacts of cultural heritage, such as the prehistoric paintings and rock art in Altamira Cave and Nerja Cave in Spain [15], and in Lascaux Cave in France [16]. Taking a broader view, the importance of aerobiology for cave ecology lies in the characterization of introduced biomass, its impacts upon cave biota, survival rate, and dispersal limitation, and any interaction with the cave-autochtonous microbes. The study provided estimates of the qualitative and quantitative impact of tourists on cave air, and the impact of natural aerosolization driven by the underground river upon the total airborne biomass in a large cave system.

2. Materials and Methods

2.1. Cave Description and Sites of Bioaerosol Impacting

Within the Classical Karst of Slovenia, the Škocjan Caves (Škocjanske jame, 45°39′53.33 N 13°59′40.44 E) are part of a complex geomorphological feature composed of four caves and deep, vertical-sided, collapse dolines formed by the Reka River [17–19]. The Reka River drains water from a surface catchment of more than 350 km^2. Its average discharge upstream of its sink into the Škocjan Cave is 8.95 m^3/s, but during floods it can reach 387 m^3/s. From the Škocjan Caves, the Reka River flows underground through the karst massif towards the Timavo spring, some 35 km away in Italy. The Škocjan Caves are 6.2 km long and 250 m deep, with large galleries and chambers, and the underground canyon carrying the Reka River [20]. They are formed in Cretaceous and Paleocene limestone, close to the contact with impermeable Eocene flysch rocks that consist of quartz-sandstone, conglomerate and marl [21]. Canyon passages have developed in massive and thick-bedded limestones, mostly along just a few tectonized bedding planes and along distinct fractured zones [22].

The site is on the UNESCO World Heritage List and is recognized as an underground karst wetland under the Ramsar Wetland Classification System. More than 5000 individuals of twelve different bat species have been identified in the Škocjan Caves [23]. A 2.6 km section of the Škocjan Caves is open to tourists throughout the year and attracts approximately 140,000 visitors per year. The number of organized tourist visits during the year depends on the season. Eight regular visits are organized during the peak season (June to September), four in the shoulder season (April, May, October) and two during the off-season (January to March, November and December). In contrast

with the dry passages of the tourist route (Tiha jama), the river canyon (Šumeča jama) is subject to flooding and aerosolization by the Reka River (Figure 1).

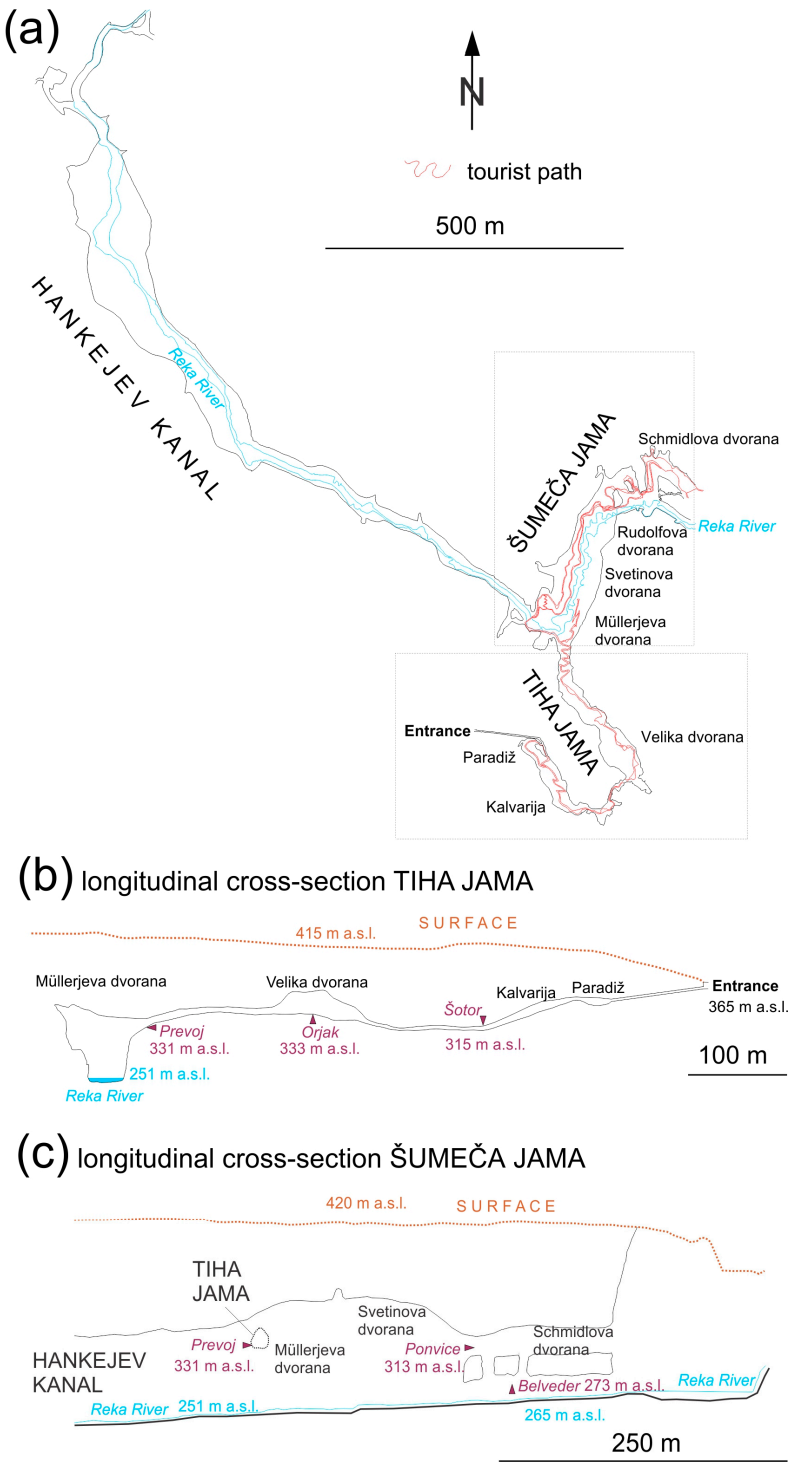

Figure 1. Map of the Škocjan Caves (**a**) with the designated locations of bioaerosol sampling sites in the Tiha jama section (Šotor, Orjak) along the tourist footpath (tourist impact, (**b**)); and in the Šumeča jama section (Prevoj, Ponvice, Belveder, with the Reka River impact, (**c**)). The ground plan is from the Cave cadastre of the Karst Research Institute ZRC SAZU (drawn by Franjo Drole).

Due to the specific cave morphology in the Tiha jama section, bioaerosols deriving from tourists provide most of the allochtonous input to the cave air whereas, in the Šumeča jama section, the impact on the cave air is attributed mainly to the Reka River (Figure 1). Approximately five metres from the tourist footpath in Tiha jama, air was sampled at a site named Šotor at the end of a large chamber (Kalvarija) with an estimated volume of 23,144 m^3 (22 January 2012, 1 April 2012, 3 June 2012, 22 July 2012, 22 August 2012, 26 September 2013, 3 December 2013, 1 July 2014, and 11 November 2014). Additionally, in the Tiha jama section, an additional site close to a stalagmite known as "Orjak" was sampled (13 November 2014) in the Velika dvorana chamber (estimated volume: 64,960 m^3).

To obtain details of the background concentration of airborne bacteria, each day's air sampling started one hour before the cave was open for tourists, and continued up to 1.5 h after the final tourist group had passed the sampling site. During the high tourist season, several tourist groups usually followed each other through the cave. The number of passing tourists were recorded.

In the Šumeča jama section of the cave, three sampling sites were selected to obtain a vertical transect above the Reka River: Belveder (Be)—the closest to the Reka River (~8 m); Ponvice (Po)—40 m above Belveder; and Prevoj (Pr) at the entry point to the Šumeča jama section, 58 m above Be (Figure 1). On 12 November 2014, the discharge of the Reka River upstream of its ponor was increasing (165–208 m^3/s), on 27 August 2015 it was in a recession period (0.201–0.385 m^3/s), as was also the case on 23 November 2016 (22.3–22.9 m^3/s). Data on discharge were provided by the Slovenian Environment Agency, from the national hydrological network on the Reka River upstream of its ponor for the Reka-Cerkvenikov mlin station and the Reka-Škocjan station [24]).

2.2. Atmospheric Conditions and Cultivation of Airborne Bacteria

Temperature was measured continuously (with a data acquisition period of 30 s) before and during tourist visits using a portable Kestrel 4500 PocketWeather Tracker (Boothwyn, PA, USA) (accuracy: ±1.0 °C; range: 45 to 125 °C), and atmospheric carbon dioxide was measured continuously (with a data acquisition period of 30 s) by a MI70 Vaisala handheld carbon dioxide metre (Vaisala Oyj, Helsinki, Finland) (probe: GMP222; accuracy: ±1.5% of range; range: 0–3000 ppm). Simultaneously with these measurements at the site, a Mas-100 Air Sample Device (Merck, Darmstadt, Germany) (with a constant flow of 100 L/min) was used to inoculate microbiological media with 500 or 1000 L of air. Bioaerosols were sampled 1.5 m above ground level using a portable platform. The perforated plate of the impactor was sterilized with 96% ethanol before inoculating microbiological media. Impacting of the air onto Petri plates with the microbiological media was carried out continuously by changing plates, and lasted up to six hours. Sampling started a few hours before the tourist visits, continued during the passing of the tourist groups, and even after tourists vacated the underground chamber. To minimise the impact of the research team, the operator was not present at the sampling location.

A 1.5% nutrient agar (NA, Sigma-Aldrich, St. Louis, MO, USA) was used to estimate and characterize the culturable airborne bacteria, because it is commonly used in microbiology, and it ensured compatibility with previous studies [4,5]. No antimicrobial substances were added to the media. After impacting, the NA plates were cultivated at 37 ± 1 °C for 24 h. Counted colony-forming units (CFU) values were corrected statistically according to Feller [25] and expressed as CFU per cubic metre. For MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) identification, distinct morphotypes from the NA plates (sampled at Šotor on 1 July 2014, 11 November 2014, and 12 November 2014; at Orjak on 13 November 2014; and in the Šumeča jama section on 12 November 2014) were subsequently inoculated on 5.0% defibrinated sheep-blood agar (BA) and incubated at 37 ± 1 °C for 24 to 48 h. BA was prepared immediate before use at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana: per 1000 mL: 15 g agar (Sigma-Aldrich), brain-heart infusion broth (Becton Dickinson, Sparks, MD, USA), and 50 mL defibrinated reoxygenated sheep blood (BioGnost, Zagreb, Croatia). Pure cultures from BA never older than 48 h were subjected to MALDI-TOF MS identification. BA is an enriched medium that allows growth of the majority of medically-important bacteria.

2.3. Characterisation of Isolates

MALDI-TOF MS is an emerging method for microbial identification, characterisation, and typing, particularly in clinical microbiology [26,27]. Outside routine diagnostics it is used for epidemiological studies, typing, and detection of antimicrobial resistance and biological warfare agents. The sample—a bacterial colony or lysed bacterial cells—is overlaid with a photo-absorptive matrix that co-crystalizes with the sample and enables the initial ionisation step. The most frequently used matrices in microbiology are α-cyano-4-hydroxy-cinnamic acid (HCCA), 2,5-dihydroxy benzoic acid (DHB), and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid). The separation of ionised molecules is based on the mass-to-charge ratio (m/z), which is represented in a graph plotting m/z and the relative intensity. The obtained mass spectrum of an unknown microorganism is compared to the mass spectra in the database. For species level identification, a mass range m/z of 2–20 kDa is used, which represents mainly ribosomal proteins, along with a few housekeeping proteins [26–28]. Matches of peak positions and their intensities in the mentioned mass range between sample and database spectra are used to generate a match score [27]. Pre-treatment protocols (formic acid extraction, formic acid—ethanol extraction etc.) are needed in cases when extraction of intracellular proteins by matrix alone is not sufficient. The greatest limitation of MALDI-TOF MS is the size of the spectral database, because identification of the isolate is only possible if the database contains the specific type of spectrum [26].

Bacterial isolates on BA were subjected to identification using MALDI-TOF MS with an on-target formic acid extraction technique. A colony was smeared onto the MALDI steel plate and overlaid with 1 µL of 70% formic acid. After drying, the sample was overlaid with 1 µL of saturated α-cyano-4-hydroxy-cinnamic acid (HCCA) matrix solution in 50% acetonitrile-2.5% trifluoroacetic acid (Bruker Daltonik, Bremen, Germany) and left to dry before subsequent analysis with Bruker MALDI Biotyper RTC software version 3.1 (Bruker Daltonik, Bremen, Germany). The Bruker bacterial test standard (Bruker Daltonik) was used for calibration according to the manufacturer's instructions. The quality of identification was assessed using the manufacturer's score value. A score of $\geq$2.000 indicated reliable species level identification, a score of 1.700 to 1.999 indicated reliable identification to the genus level, and a score of <1.700 was interpreted as no identification. Microbial risk groups were assessed according to the Risk Group Database of the American Biological Safety Association [29].

2.4. Statistical Evaluation

Statistical analyses were performed using PAST [30] for species accumulation curve and Daniel's XL Toolbox, an open-source add-in for Microsoft Excel (Version 6.60, licensed under the Apache License, Version 2.0, Daniel Kraus, Würzburg, Germany) for linear regression.

3. Results and Discussion

3.1. Tourist-Derived Cave Air Biomass

A previous study on aerosol nanoparticles revealed that the Škocjan Caves are well ventilated with notable impacts both from polluted atmospheric air and from tourist presence, particularly in summer [31]. In the current study, the presence of tourists did not inevitably reflect as a clear peak of carbon dioxide (e.g., 22 July 2012, 22 August 2012, 26 September 2013, 11 November 2014, Table 1) that could be attributed to the specificity of the selected microlocation and the impact of the external climate [5]. Additionally, the highest measured concentration of carbon dioxide (2480 ppm) did not correspond to the highest number of visitors (1 July 2014). In caves, the concentration of carbon dioxide is generally lower during the winter, reaching a peak during the summer [32]. The concentration of airborne bacteria during tourist visits always exceeded the bacterial level before the tourists' presence (Table 1). The lowest concentration of airborne bacteria was recorded when the Tiha jama section was closed to tourists for 44 days (3 December 2013). This value can be regarded as an approximation of the cave air biomass without any tourist influence.

Table 1. Air temperature, concentrations of carbon dioxide, and airborne bacteria at the Šotor site (Tiha jama) before and after the arrival of tourists, with the number of tourists passing the sampling station; data from 2012 compiled from the previous study [5].

Sampling	Climate Condition	Before Tourists		After Tourists		
Date	Temp. (°C)	CO_2 (ppm)	Bacteria (CFU/m^3)	CO_2 (ppm)	Bacteria (CFU/m^3)	Tourist Count
22 January 2012	12.5	437 ± 11	11 ± 2			0
min/max		410 460	8 15			
1 April 2012	12.7	593 ± 12	11 ± 19	625 ± 13	71 ± 32	95
min/max		560 620	1 54	580 560	25 114	
3 June 2012	13.0	1258 ± 17	8 ± 4	1284 ± 24	256 ± 138	281
min/max		1210 1310	2 13	1230 1340	78 558	
22 July 2012	13.0	2007 ± 38	91 ± 34	1957 ± 50	382 ± 253	310
min/max		1900 2100	41 138	1820 2040	74 1008	
22 August 2012	13.4	1062 ± 43	19 ± 7	1040 ± 49	250 ± 87	296
min/max		960 1170	10 30	920 1160	80 438	
26 September 2013	12.9	1367 ± 21	10 ± 4	1290 ± 49	152 ± 75	265
min/max		1310 1420	4 16	1150 1310	46 394	
3 December 2013	12.6	453 ± 10	2 ± 2	†	†	†
min/max		430 480	0 6			
1 July 2014	12.9	2402 ± 25	5 ± 3 *	2281 ± 74	24 ± 19 *	143
min/max		2340 2480	1 11	2140 2340	8 72	
11 November 2014	12.7	2075 ± 21	19 ± 13	2069 ± 23	22 ± 11 *	27
min/max		1950 2120	9 40	1950 2150	9 44	
12 November 2014	nd	nd	nd	2256 ± 24	37 ± 28	50
min/max	12.5			2200 2310	9 63	

†—cave section closed to tourists from 18 November to 31 December 2013. *—isolates subjected to MALDI-TOF MS identification, see Tables 2–4. nd—no data.

The biomass indicator of cultivable airborne microorganisms that was employed clearly pointed out their human source during tourist visits. After tourists reached the sampling station there was a statistically significant strong positive correlation between the number of tourists and the increase of the concentration of airborne bacteria above the background level (Figure 2). Taking into consideration the recommended limiting value of 300 CFU/m^3 for total microbial counts for indoor commercial and residential environments [33], the linear relationship between the increase in the concentration of airborne bacteria and the tourist counts, the impact of just 370 tourists could raise microbe levels to the limiting value.

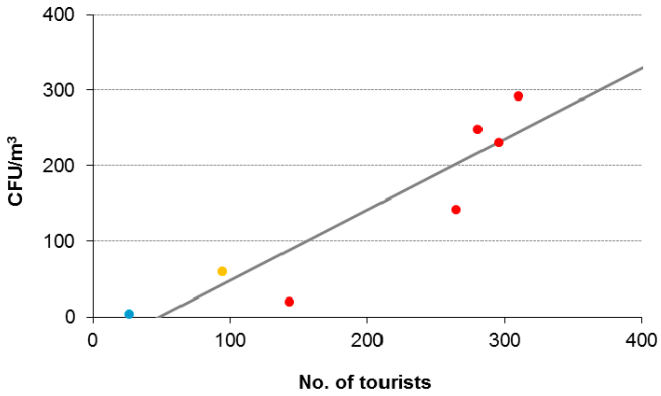

Figure 2. Relationship between tourist counts (n = 7) and the increase in concentration of airborne bacteria from their background level at the Šotor sampling site (increase in biomass expressed in CFU/m^3 = 0.94 × tourist count—45.28; p = 0.001; blue—off-season, orange—shoulder season, red—peak season; see Material and Methods and Table 1 for details).

After tourists vacated the underground chamber, the concentration of airborne bacteria slowly dropped due to air circulation. In such large and relatively open cave systems, natural air movements and the circulation induced by heat emission from lamps enhance the translocation of minute particles, but the question of dispersal limitation in such conditions remains open. It seems that, generally, dispersal limitation can be a key process in structuring the air microbiome [34]. Both biomass and biomass diversity, particularly of fungi, are higher in small and/or closed underground chambers [6]. In the comparatively far smaller Altamira Cave with its Palaeolithic paintings, a notable level of bacteria related to humans was reported, and the use of protective gloves, masks, and clothing was recommended in considering the case for re-opening the cave to the public [13]. For such relatively small and highly vulnerable sites, with rock art susceptible to biodeterioration, there was even a proposal for a tentative index of fungal hazard [14].

3.2. Diversity of Human-Related Airborne Bacteria

Altogether, 868 isolates were subjected to identification by MALDI-TOF MS and 88 (~10%) were discarded from further analysis by the Biotyper RTC software. Two-hundred and eleven processed isolates were from periods preceding tourist presence and 569 were from periods of tourist presence. For both groups, a similar proportion of colonies were identified at species level (no tourists—23.7%; with tourists—21.1%) and at the genus level (no tourists—26.5%; with tourists—28.8%), with a large proportion of unidentified colonies (no tourists—49.8%; with tourists—50.1%).

Several airborne bacteria were identified solely in the air sampled before tourist visits, and some of them might represent the natural background without direct tourist impact: *Arthrobacter arilaitensis*, *Kocuria polaris*, *Paenibacillus amyolyticus*, *P. polymixa*, *Pseudomonas antarctica*, *P. cedrina* ssp. *cedrina*, *P. jessenii*, *P. marginalis*, *Staphylococcus equorum*, *S. haemolyticus*, *S. pasteuri*, *S. warneri*, and *Streptomyces badius* (Table 2). More diversified microbiota were sampled during tourist visits (Tables 2 and 3). The presence of some airborne microbes might also be associated with the presence of bats, because the Škocjan Caves host several colonies, exceeding 5000 individuals, year-round [35]. In a study of Ardales Show Cave (Spain), with its Palaeolithic paintings, the majority of the culturable airborne bacteria were Gram-positive before (*Streptomyces*), and even after, a visit (*Bacillus*, *Streptomyces*). *Bacillus* spp. were frequently present, but with a lower abundance during the cave closure periods. Furthermore, some bacteria, e.g., *Arthrobacter*, *Micrococcus*, *Pseudomonas*, showed no relevant patterns in their distribution [8].

Table 2. Identification of airborne bacterial isolates (MALDI-TOF MS log score value >2.000, species level identification) at Šotor and Orjak (*) sampling station before and during tourist visits, with the number of passing tourists (in brackets) in the Šumeča jama section during high Reka River discharge (Δ).

Species	1 July 2014 (0)	1 July 2014 (143)	11 November 2014 (0)	11 November 2014 (27)	12 November 2014 (0)	13 November 2014 * (0)	12 November 2014 Δ
Acidovorax facilis	.	.	.	+	.	.	.
Acinetobacter haemolyticus	.	.	.	+	.	.	.
Acinetobacter johnsonii	.	+	.	.	.	.	.
Acinetobacter junii	.	.	.	+	.	.	.
Acinetobacter parvus	.	.	.	+	.	.	.
Acinetobacter tjenbergiae	.	.	.	+	.	.	.
Aerococcus viridans	+	+	.	.	.	+	.
Aeromonas encheleia	.	.	.	.	.	.	+
Arthrobacter arilaitensis	+	.	+	.	.	.	.
Bacillus cereus	.	.	.	.	.	.	+
Bacillus subtilis ssp. *subtilis*	.	+	.	.	.	.	.
Bacillus marisflavi	.	.	.	.	.	.	+
Bacillus thuringensis	.	.	.	+	.	.	.
Brachybacterium faecium	.	.	.	+	.	.	.
Brevundimonas diminuta	.	.	.	+	.	.	+
Dietzia maris	.	+	.	.	+	.	.

Table 2. *Cont.*

Species	1 July 2014 (0)	1 July 2014 (143)	11 November 2014 (0)	11 November 2014 (27)	12 November 2014 (0)	13 November 2014 * (0)	12 November 2014 Δ
Kocuria carniphila	.	.	.	+	.	.	.
Kocuria polaris	.	.	.	.	+	.	.
Kocuria rosea	.	.	.	+	.	+	.
Kytococcus sedentarius	.	.	.	+	.	.	.
Microbacterium hydrocarbonoxydans	.	.	.	+	.	.	.
Microbacterium sp.	.	+	.	.	.	.	.
Micrococcus luteus	.	+	.	+	+	+	.
Micrococcus terreus	.	.	.	+	.	.	.
Paenibacillus amyolyticus	+	.	+	.	.	.	.
Paenibacillus polymixa	+	.	.	.	.	.	.
Pseudochrobactrum asaccharolyticum	.	.	.	+	.	.	.
Pseudoclavibacter helvolus	.	+	.	.	.	.	.
Pseudomonas antarctica	.	.	+	.	.	.	.
Pseudomonas cedrina ssp. *cedrina*	.	.	+	.	.	.	.
Pseudomonas extremorientalis	.	.	.	.	.	.	+
Pseudomonas jessenii	.	.	+	.	.	.	.
Pseudomonas marginalis	.	.	+	.	.	.	.
Pseudomonas synxantha	.	.	.	.	.	.	+
Psyhcrobacillus psychrotolerans	+	+	+	.	.	+	.
Rhodococcus erythropolis	.	.	.	+	.	.	.
Staphylococcus capitis	.	.	.	+	.	.	.
Staphylococcus capitis ssp. *capitis*	.	.	.	+	.	.	.
Staphylococcus epidermidis	+	.	+	+	.	.	.
Staphylococcus equorum	.	.	.	.	+	+	.
Staphylococcus equorum ssp. *equorum*	.	.	.	+	.	.	.
Staphylococcus haemolyticus	.	.	.	.	+	.	.
Staphylococcus pasteuri	.	.	.	.	.	+	.
Staphylococcus warneri	.	.	.	.	+	.	.
Staphylococcus xylosus	.	.	.	+	.	+	.
Streptomyces badius	+	.	+	.	.	.	.
Total identified species	7	8	9	21	6	7	6

.—absence; +—presence.

Table 3. Identification of airborne bacterial isolates (MALDI-TOF MS log score values of 1.700–1.999, genus level identification) at Šotor and Orjak (*) sampling stations before and during tourist visits with the number of passing tourists (in brackets) in the Šumeča jama section during the high Reka River discharge (Δ).

Species	1 July 2014 (0)	1 July 2014 (143)	11 November 2014 (0)	11 November 2014 (27)	12. November 2014 (0)	13 November 2014 * (0)	12 November 2014 Δ
Acinetobacter calcoaceticus	.	.	.	.	+	.	.
Acinetobacter haemolyticus	.	+	.	.	+	.	.
Acinetobacter haemolytius	.	.	.	+	.	.	.
Acinetobacter junii	.	+	.	+	.	.	.
Acinetobacter lwoffii	.	.	.	.	.	.	+
Acinetobacter parvus	.	.	.	+	.	.	.
Acinetobacter tjernbergiae	.	.	.	+	.	.	.
Aerococcus viridans	+	+	+	+	.	+	.
Arthrobacter arilaitensis	+	.	+	.	.	+	.
Bacillus arsenicus	.	.	.	+	.	.	.
Bacillus cereus	.	.	.	.	+	.	+
Bacillus flexus	.	+	.	.	.	.	.
Bacillus jeotgali	+	.	.	.	.	.	.
Bacillus licheniformis	.	+	.	.	.	.	+
Bacillus muralis	.	+	.	.	.	+	.
Bacillus pumilus	+	+	+	.	.	.	.
Bacillus simplex	.	+	.	.	+	+	.
Bacillus subtilis ssp. *subtilis*	.	+	.	.	.	.	.
Bacillus thuringiensis	.	.	.	.	.	+	+
Brachybacterium faecium	.	.	.	.	.	+	.
Brevundimonas diminuta	.	.	.	+	.	.	+
Carnobacterium maltaromaticum	.	.	.	.	.	.	+
Dietzia cinnamea	.	.	.	+	.	.	.

Table 3. *Cont.*

Species	1 July 2014 (0)	1 July 2014 (143)	11 November 2014 (0)	11 November 2014 (27)	12. November 2014 (0)	13 November 2014 * (0)	12 November 2014 Δ
Dietzia natronolimnaea	.	+	.	.	.	.	.
Jeotgalicoccus halotolerans	.	.	.	+	.	.	.
Kocuria carniphila	.	.	.	+	.	+	.
Kocuria rhizophila	.	.	.	+	.	.	.
Kocuria rosea	.	.	.	.	.	+	.
Kyotococcus sedentarius	.	.	.	+	.	.	.
Micrococcus luteus	.	.	.	+	.	+	.
Micrococcus terreus	.	.	.	+	.	.	.
Paenibacillus polymixa	+	.	.	.	.	.	.
Pseudochrobactrum asaccharolyticum	.	.	.	+	.	.	.
Pseudomonas anguilliseptica	.	.	.	.	+	.	.
Pseudomonas azotoformans	.	.	+	.	.	.	.
Pseudomonas chlororaphis	.	.	.	.	+	.	.
Pseudomonas frederiksbergensis	.	.	.	.	.	.	+
Pseudomonas libanensis	.	.	.	.	.	.	+
Pseudomonas marginalis	.	.	+	.	.	.	.
Pseudomonas rhodesiae	.	.	+	.	.	.	.
Pseudomonas synxantha	.	.	.	.	.	.	+
Pseudomonas syringae ssp. *syringae*	.	.	+	.	.	.	.
Pseudomonas tolaasii	.	.	+	.	.	.	.
Psyhcrobacillus psychrotolerans	.	+	.	.	.	+	.
Rhodococcus erythropolis	.	.	.	.	.	+	.
Rhodococcus epidermidis	.	.	.	.	.	+	.
Staphylococcus equorum	.	.	.	+	+	+	.
Staphylococcus equorum ssp. *equorum*	.	.	.	+	.	.	.
Staphylococcus hominis ssp. *hominis*	.	.	.	+	.	.	.
Staphylococcus warneri	.	.	.	+	.	.	.
Staphylococcus xylosus	.	.	.	+	.	.	.
Stenotrophomonas rhizophila	.	.	.	.	.	.	+
Total identified genera	5	11	8	20	7	13	10

.—absence; +—presence.

Some of the identified airborne bacterial species are not directly related with the human microbiome (e.g., *Acidovorax facilis*, *Kocuria polaris*, *Microbacterium hydrocarbonoxydans*, *Paenibacillus polymyxa*, *Pseudomonas antarctica*, *Psychrobacillus psychrotolerans*, *Rhodococcus erythropolis*, *Streptomyces badius*) and are typical inhabitants of soil and aquatic environments. On the other hand, some belong to a typically human and animal biota, e.g., *Acinetobacter haemolyticus*, *A. johnsonii*, *A. junii*, *A. parvus*, *Dietzia maris*, *Pseudochrobactrum asaccharolyticum*, *Staphylococcus capitis*, *S. epidermidis*, and *S. haemolyticus*, *S. warneri*, Table 4).

Based on various national criteria (e.g., Australia, Belgium, Switzerland, Germany, New Zealand, Singapore, and the United Kingdom, Table 4) for assessment of microbial risk groups available at the American Biological Safety Association, few isolates were attributed to Risk Group 2 (organisms that can cause disease in humans, but the disease is treatable, Table 4). Their entry point is usually by inhalation of aerosolized cells and spores or direct contact with contaminated surfaces. These identified microorganisms are associated with the normal human microbiome and can cause disease only in patients that have a pronounced susceptibility to opportunistic infections.

Table 4. Characteristics of identified bacterial species.

Species	Phylum	Optimum Growth Temperature (°C)	Typical Habitat (Pathogenicity) *	Risk Group (Country)
Acidovorax facilis	Proteobacteria	30	Soil	
Acinetobacter haemolyticus	Proteobacteria	28	human skin (rarely pathogen)	
Acinetobacter johnsonii	Proteobacteria	26	humans, animals, activated sludge, food (pathogen)	2 (AU, CH, NZ)
Acinetobacter junii	Proteobacteria	28	ubiquitous, humans (pathogen)	2 (AU, CH, NZ)
Acinetobacter parvus	Proteobacteria	35	Humans	
Acinetobacter tjernbergiae	Proteobacteria	30	aquatic, wastewater	
Aerococcus viridans	Firmicutes	30	ubiquitous, air, humans (pathogen)	2 (CH, DE)
Aeromonas encheleia	Proteobacteria	28	aquatic, humans, animals	
Arthrobacter arilaitensis	Actinobacteria	10–30	food, cheeses	
Bacillus cereus	Firmicutes	20–40	ubiquitous (pathogen)	2 (AU, CH, DE, NZ, SG, UK)
Bacillus subtilis	Firmicutes	25–35	Ubiquitous	
Bacillus marisflavi	Firmicutes	30–37	marine tide zone	
Bacillus thuringiensis	Firmicutes	30–37	ubiquitous, (pathogen)	
Brachybacterium faecium	Actinobacteria	25–30	soil, feaces	
Brevundimonas diminuta	Proteobacteria	30–35	ubiquitous (pathogen)	2 (CH, DE)
Dietzia maris	Actinobacteria	26	humans, animals (pathogen)	
Kocuria carniphila	Actinobacteria	28–37	food, meat	
Kocuria polaris	Actinobacteria	20	Aquatic	
Kocuria rosea	Actinobacteria	25–37	soil, aquatic (opportunistic pathogen)	
Kytococcus sedentarius	Actinobacteria	25–37	aquatic, human skin (pathogen)	
Microbacterium hydrocarbonoxydans	Actinobacteria	30–37	crude oil	
Micrococcus luteus	Actinobacteria	25–37	soil, aquatic, air, human and animal skin	
Micrococcus terreus	Actinobacteria	33	Soil	
Paenibacillus amylolyticus	Firmicutes	28–37	Soil	
Paenibacillus polymyxa	Firmicutes	30	soil, marine sediment, plant roots	
Pseudochrobactrum asaccharolyticum	Proteobacteria	20–30	Humans	
Pseudoclavibacter helvolus	Actinobacteria	28–30	food, butter	
Pseudomonas antarctica	Proteobacteria	22	aquatic, cyanobacterial mats	
Pseudomonas cedrina	Proteobacteria	21–28	Aquatic	
Pseudomonas extremorientalis	Proteobacteria	30	Aquatic	
Pseudomonas jessenii	Proteobacteria	25–30	aquatic (opportunistic pathogen)	
Pseudomonas marginalis	Proteobacteria	28–30	plants (plant pathogen)	
Pseudomonas synxantha	Proteobacteria	25–30	Plants	
Psychrobacillus psychrotolerans	Firmicutes	25	Soil	
Rhodococcus erythropolis	Actinobacteria	20	Soil	
Staphylococcus capitis	Firmicutes	30–40	human skin (opportunistic pathogen)	
Staphylococcus epidermidis	Firmicutes	26–37	human and animal skin (pathogen)	2 (BE, CH, DE)
Staphylococcus equorum	Firmicutes	30	human and animal skin, food, sausages, cheeses	
Staphylococcus haemolyticus	Firmicutes	34–35	human and animal skin (pathogen)	2 (BE, CH, DE)
Staphylococcus pasteuri	Firmicutes	35–37	humans, animals, food (pathogen)	2 (BE, CH)
Staphylococcus warneri	Firmicutes	30–40	human and animal skin	
Staphylococcus xylosus	Firmicutes	25–35	ubiquitous, human and animal skin	
Streptomyces badius	Actinobacteria	28	Soil	

*—various sources (e.g., [36–42]); AU—Australia, BE—Belgium, CH—Switzerland, DE—Germany, NZ—New Zealand, SG—Singapore, UK—United Kingdom.

The diversity of airborne bacteria revealed by MALDI-TOF MS was not high; this is related directly to the limited availability in the database of mass spectra for environmental isolates, including those from caves [43] and the selected medium and cultivation conditions. A sample rarefaction analysis confirmed that only a small proportion of the airborne bacteria was revealed in the Tiha jama section during the 2014 sampling campaign (Figure 3).

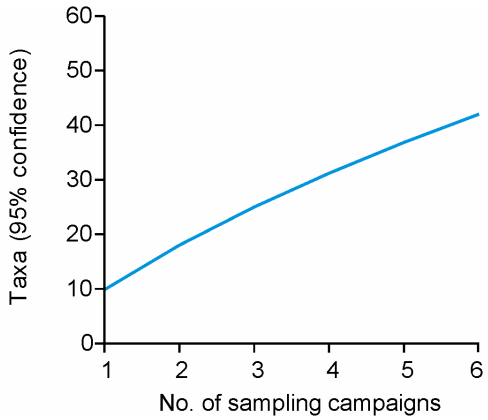

Figure 3. Estimated bacterial species richness after six sampling campaigns at the Šotor and Orjak sampling stations before, and during, tourist visits in 2014.

3.3. Attribution of River Aerosolization to Airborne Biomass

The Škocjan Caves are also an excellent example for underground, naturally-induced, aerosol formation due to splashing and mist deriving from the underground river. Large open spaces along the Reka River canyon (Figure 1A,C) are reflected in large variations of temperature and carbon dioxide among the sampling sites within a vertical transect (Figure 4). In the Šumeča jama section (Figure 1), the important factors affecting the air temperature are the temperature of the Reka River, its discharge, and the distance from the cave entrance and vertical position within a cave transect [44]. In comparison to past organic pollution of the Reka River [45], the situation is, nowadays, improved although there are some recorded events with elevated indicators of microbial fecal pollution (unpublished data).

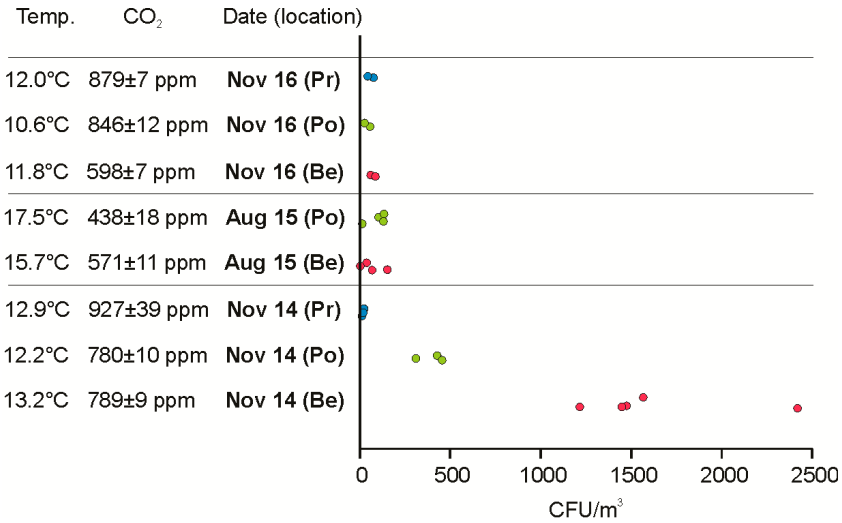

Figure 4. Atmospheric conditions and concentrations of airborne bacteria at three sampling locations Belveder (Be), Ponvice (Po), and Prevoj (Pr) along the underground channel of the Reka River, with different discharges in November 2014 (~200 m^3/s), August 2015 (~0.2 m^3/s), and November 2016 (~20 m^3/s).

The high discharge of the Reka River (~200 m^3/s) was responsible for an elevated aerosolization rate of bacteria from the river and consequently high concentration of airborne bacteria close to the river (Belveder site with the highest concentration of 2418 CFU/m^3, Figure 4). The impact of the river in a vertical transect above the Belveder site gradually diminished (the highest concentration at the Ponvice station was 455 CFU/m^3 and that at the Prevoj station 23 CFU/m^3, 12 November 2014, Figures 1 and 4). In the air, six bacterial species (Table 2) and an additional six genera (*Acinetobacter*, *Bacillus*, *Brevundimonas*, *Carnobacterium*, *Pseudomonas*, *Stenotrophomonas*, Table 3) were identified. *B. cereus* was dominant at the Belveder and Ponvice sampling stations where *P. extremorientalis* was also particularly abundant.

During an approximately 10-times lower discharge episode in the autumn of 2016, the concentration of airborne bacteria at the Belveder site was lower (~20-times), but within a similar range to that on 27 August 2015, when the Reka River had low discharge and air temperatures were far higher (Figure 4). It is likely that not only the discharge influences the concentration of airborne bacteria, but also other atmospheric conditions, such as temperature. For some microbes replication in the air was already proven [46], and caves lacking UV and desiccation stress, and with high levels of subcellular minute particles and organic molecules, can be supportive of microbial replication.

Airborne microbes and other minute organic particles also represent a source of nutrients for cave biota. In some caves, such as the Škocjan Caves, there are three major sources of airborne bacteria, autochtonous to the underground, human- and river-derived. At a certain point these subpopulations

interact with each other (e.g., Prevoj sampling station), which introduces an additional ecological aspect to the cave ecosystem, e.g., gene transfer.

4. Conclusions

Cave air acts as a sink for externally-introduced airborne biomass that is available to the cave ecosystem. In show caves tourists are a significant vector and source of airborne bacteria. Tourist numbers correlate directly with the increase of airborne biomass, but vast cave spaces, such as those in the Škocjan Caves, prevent long-lasting on-site concentration of bacterial biomass. These aerosolized bacteria float or persist on surfaces for relatively long periods and form a reservoir for the colonization/infection of susceptible hosts. The discharge of the underground river impacts directly upon the formation of aerosols, the distribution of which in a cave is governed by cave morphology and thermal convection.

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