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Tourist Traffic Significantly Affects Microbial Communities of Sandstone Cave Sediments in the Protected Landscape Area "Labské Pískovce" (Czech Republic): Implications for Regulatory Measures

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Abstract: In the Protected Landscape Area "Labské pískovce" (Czech Republic), there are many sandstone caves accessible by permission only and where climbing equipment is commonly required. The tradition of visiting the caves dates back to turn of the 19th and 20th centuries, and visitors traditionally record their visits in log-books. We have gathered attendance data from 12 caves from log-books and via survey (2-year form collection period). The attendance varied from less than 10 to ~650 visitors annually. Signs of inadequate behavior of visitors were observed, especially waste disposal and smoking in the caves. Sediment step-compression increased significantly the ratios of fungi/bacteria and gram-positive to gram-negative (G+/G−) phospholipid fatty acids (PLFA) as well as PLFA of actinobacteria and activities of sediment phosphatases, glucosidases, and peroxidases. The number of visitors was correlated positively with the G+/G− ratio and sediment nutrients but negatively with sediment moisture and the activity of peroxidases. The results indicate a significant effect of attendance on sediment microbial communities, most likely caused by the import of nutrients into caves. Hence, reconsideration of the cave attendance policy is recommended.

Keywords: sandstone caves; "Labské Pískovce"; sediment microbial communities; phospholipid fatty acids; soil enzyme activities

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1. Introduction

Caves, unlike other underground ecosystems, are accessible to humans. The study of ecosystems of caves thus represents a unique opportunity to study underground ecosystems in general. The most attractive are extensive underground and passive systems whose environment is markedly different from the outside or from the environment of small caves. Under Central European conditions, such caves are found in limestone areas, where they are formed by the dissolution of rocks, but other rocks and mechanisms also occur, which make possible the formation of large cave systems.

The target caves originated in sandstone and are located in a large unique area, conserved for its environmental and cultural specifics, i.e., the Protected Landscape area (PLA) "Labské pískovce", or in English "Elbe Sandstone Mountains". They are part of the National Nature Reserve (NNR) Kaňon Labe (for more detail on this sandstone landform see, e.g., [1]). Although unhindered nature conservation in NNR areas is one of the priorities, and entry outside of marked pathways is prohibited (i.e., also to caves) without permission, violations commonly occur with respect to caves. However, in addition to remnants of local picnic spots in caves or apparent local damage to cave walls after climbing, we can speculate there is a larger negative impact of tourism on cave ecosystems or their components. There were several fauna studies in the caves of interest, but none answered this question clearly. In general, the caves are characterized by relatively low biodiversity as well as low abundance of plant and animal species. For example, Holec et al. [2] used pitfall traps to investigate the species spectrum, but the number of individuals in the traps was very low for statistical processing. However, the impact of tourism on the species composition can still be assumed at the arthropod level. For example, Chapman [3] noted changes in Collembolans and spider taxa in an Otter Hole cave near Chepstow, a cave area which popular as an picnic site with visiting cavers. In the case of the study of bats (Chiroptera) from the cave area of our interest, fluctuating numbers of visitors could also be related to the occurrence of bats, but for chiropterologists it was difficult to prove in less accessible and inaccessible parts of the caves [4]. In addition, these studies do not provide any quantitative data on cave attendance.

The determination of the possible effect of tourism on communities of the ecosystem can be based on the assessment of mechanisms of soil changes in association with compaction or erosion, or the accumulation of allochthonous organic matter, accumulation of artifact remnants with toxic nicotine (cigarette), phthalates (plastics), heavy metals (batteries), etc.

Caves are oligotrophic environments; available nutrients are limited. Soil (sediment) microorganisms play an essential role in nutrient cycling and general biogeochemical processes (e.g., [5]). Changes in soil microbial community composition and microbial activities can therefore affect overall ecosystem health. Microorganisms respond sensitively to different environmental changes, and analysis of microbial biodiversity and biomass can be useful for evaluation of cave features. In particular, cave sediments [6] affected by exogenous material associated with human access can serve as a good indicator of the human impact on caves [7]. For example, Ikner et al. [8] postulated that abundance of Proteobacteria along the limestone karst cave trail was affected by increased organic matter availability due to lint and other organics brought in by cave visitors. According to Chelius et al. [9], organic amendments in the form of lint and rodent feces altered both abundance and diversity of sediment bacterial communities. Those authors raised the issue of possible replacement of non-described species by even the small introduction of lint and organic matter foreign to that environment and the risk of irreversible loss of biodiversity.

Nevertheless, most of these studies were focused on cave walls, while studies on cave sediments are scarce [7]. In addition, the human impact on caves was previously studied mainly in karst caves [10]. Sandstone caves are scarcely studied for impact of human visitation. Current knowledge implies the possibility of changes in ecosystems of caves of interest, but hard data are missing, and further studies are needed. We hypothesize that high tourist activity can affect microbial communities of sandstone caves. Based on our repeated excursions to caves of interest, we predict that the most important factor responsible for the mentioned changes can be organic matter distributed on tourists' shoes.

We carried out this study to assess the effect of tourist traffic on cave microbial communities and their nutrient cycling potential. We analyzed the structure, quantity, stress indicators and activities of cave sediment microbial communities, an important ecological indicator of cave ecosystem health, of sediments of 12 caves in the PLA Labské Pískovce (Czech Republic). Microbial analyses were supplemented by analyses of available nutrients and other soil parameters. Those data were compared to the attendance data obtained from historical log-books and were confirmed by a two-year form survey carried out in the caves.

2. Materials and Methods

2.1. Sampling Localities

The area of interest lies in the NNR Kaňon Labe (Labe Canyon), and it is part of the larger PLA Labské pískovce (Elbe Sandstone Mountains). A list of analyzed caves is provided in Table 1, and Figure 1 shows the map of the locality.

The PLA Labské pískovce is connected to a similar protected area called Saxony-Switzerland (Landschaftschutzgebiet Sächsische Schweiz) in the Federal Republic of Germany. The National Park Saxon Switzerland (Nationalpark Sächsische Schweiz) was established in 1991 from the most valuable part of this German protected area. České Švýcarsko National Park in the Czech Republic was established in 2000. The typical landscape character, which is divided by deep canyon valleys and a high proportion of forest, is the basic characteristic of this protected area [11,12].

The geological basement of the entire territory is formed by a massive set of cretaceous sandstones [12,13]. Pedologically, podzols prevail in the dominant forest soils of this area [14]. Coniferous and mixed forests with dominating tree species of the genera *Pinus*, *Picea*, *Quercus*, *Carpinus* and *Fagus* (according to habitat conditions) are the accompanying plant community of these acidic nutrient poor forest soils on sands. The area is relatively cold with a tendency to the oceanic climate [12]. However, due to varied geomorphological characteristics of the area, the climate also varies.

Table 1. List of analyzed caves, localization and average attendance per year.

Cave	GPS	Annual Attendance
Cipískova jeskyně (Cipísek's cave)	50°49′44.4″ N, 14°14′03.7″ E	19.40 ± 6.73
Horní Stelzig (Upper Stelzig)	50°49′08.1″ N, 14°13′51.0″ E	<10
Jeskyně nadějí (Cave of Hope)	50°51′32.6″ N, 14°13′38.3″ E	88.00 ± 27.00
Jeskyně Otto Mortzsche (Otto Mortzsche's cave)	50°51′43.5″ N, 14°13′53.3″ E	20.20 ± 3.90
Jeskyně přátel přírody (Friends of Nature cave)	50°51′33.0″ N, 14°13′38.1″ E	162.80 ± 67.88
Jeskyně Přátelství (Friendship cave)	50°51′48.9″ N, 14°13′48.3″ E	534.60 ± 175.84
Kabinet přírodovědy (Cabinet of Nature Science)	50°50′00.3″ N, 14°14′05.8″ E	<10
Krakonošova jeskyně (Krakonoš's cave)	50°51′26.7″ N, 14°13′35.5″ E	21.40 ± 8.17
Ledová jeskyně (Icy cave)	50°51′49.8″ N, 14°13′47.0″ E	116.60 ± 46.82
Loupežnická jeskyně (Highwayman cave)	50°51′09.7″ N, 14°13′23.0″ E	649.60 ± 47.69
Máslová díra (Butter hole)	50°51′29.0″ N, 14°13′38.3″ E	27.20 ± 15.55
Rytířský sklep (Chevalier cellar)	50°49′06.7″ N, 14°13′52.7″ E	28.60 ± 21.30

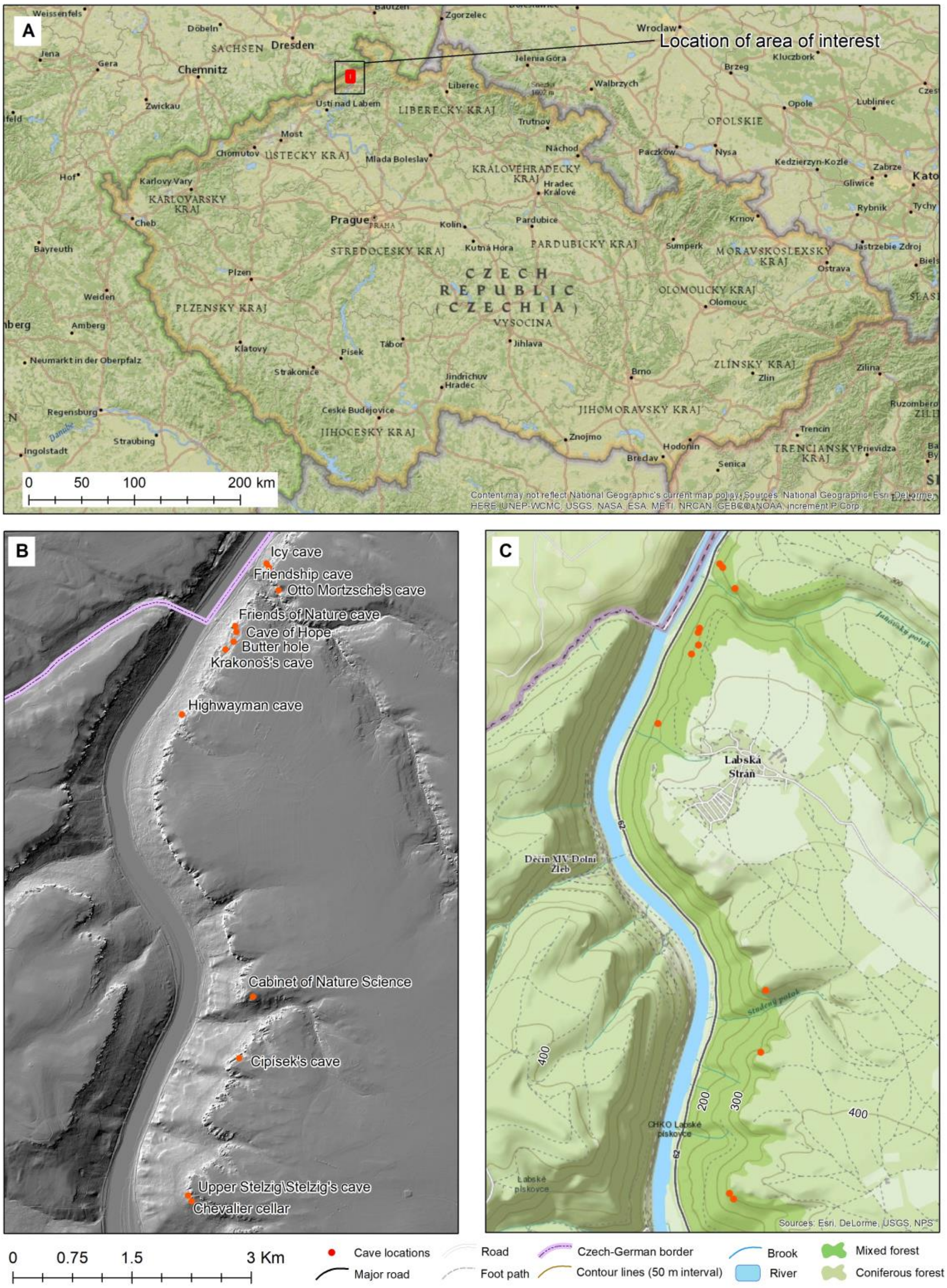

Figure 1. Location of the analyzed caves in the Labe (Elbe) river canyon; (**A**) Localization of the study area within central Europe; (**B**) Digital elevation model of the Labe (Elbe) river canyon (**C**) Topographical map of the Labe (Elbe) canyon.

2.2. Analyses of Attendance

Attendance of caves was determined from log-books, which are located in all caves in the area. The history of the log-books dates back to beginning of the 20th century (see Appendix A for more details), to the beginning of speleological interest in the area. Log-books are predominantly located at the deep end of the caves. Since the access to log-books is not simple and often requires climbing equipment and good physical status, it is considered an honor to log in a book. As a result, the records in the log-book present quite reliable source of data, especially for assessment of differences in attendance of different caves. Absolute values can be considered a low estimate (minimal confirmed value) of the attendance.

The log-books records from calendar years 2008–2012, prior to sampling, were collected. We have chosen this time period as it is the most representative of recent impact of attendance on cave ecosystems and because most complex attendance data are available. To confirm the data from log-books, a form-survey was carried out prior to sampling from May 2011 to May 2013. The forms were prepared in three languages (Czech, German, and English; see supplementary Figure S1 for example). The questionnaires included 14 questions in total relating to basic demographic data, the visitor's motivation and classification in the context of speleotourism. Filled forms were collected each month (and empty questionnaires were replenished).

The attendance data from log-books are presented in Table 1; demographic data obtained from form-survey are presented as Appendix B.

2.3. Sampling

In total, 12 caves were selected for observation (Table 1). These differed in their attractiveness for visitors as was roughly known by advance study from log-books. Attendance ranged from < 10 to ~650 visitors/year. In each of the caves, sediment samples were taken along the gradient of the walkway from the entrance. The number of samples taken in each cave was proportional to the size of the cave, but it was also dependent on the availability of suitable sampling points because sediments were missing in some parts of the caves. Two types of samples were collected: samples affected by trampling in the part of cave where the majority of visitors step, and non-affected in the part where stepping is unlikely.

The amount of available sediments was generally limited. In order to limit the effect of the research on the sediment ecosystems, the amount of withdrawn sample was limited and in several cases did not allow carrying out all nutrient analyses. Microbial analyses, i.e., PLFA and enzyme activities, were carried out as priority. Statistical evaluation was appropriately adapted to this fact (see Section 2.6 for details).

2.4. Microbial Analyses

2.4.1. Determination of PLFA

PLFA determination was carried out by adopted method of Zelles et al. [15] corresponding to ISO 29843-2 [16] used in our previous studies [17–19]. Briefly, total lipids were extracted from sediments using a single-phase mixture of methanol, chloroform and phosphate buffer (2:1:0.8). The polar lipid fraction (involving phospholipids) was then isolated on silica columns (Supelclean^{TM} LC-Si, Sigma-Aldrich, St. Louis, MO, USA), methanolyzed and analyzed by means of GC-MS.

Total PLFA was quantified using internal standard methyl nonadecanoate. Living microbial biomass was estimated based on the total PLFA content ($PLFA_{tot}$). The biomass of dominant microbial groups was estimated according to [20]; i.e., fungal biomass ($PLFA_{fun}$) as 18:2ω6,9, bacterial biomass ($PLFA_{bac}$) as the sum of i14:0, i15:0, a15:0, 16:1ω7t, 16:1ω9, 16:1ω7, 10Me-16:0, 17:0, i17:0, a17:0, cy17:0, 10Me-17:0, 18:1ω7, 10Me-18:0, cy19:0, biomass of Gram-positive bacteria ($PLFA_{G+}$) as the sum of i14:0, i15:0, a15:0, i17:0, a17:0 and biomass of Gram-negative bacteria ($PLFA_{G-}$) as the sum of cy17:0, cy19:0 and 18:1ω7 and biomass of actinobacteria ($PLFA_{ac}$) as the sum of 10Me-16:0, 10Me-17:0, 10Me-18:0. Ratios of fungal to bacterial biomass and Gram-positive to Gram-negative biomass were estimated as $PLFA_{fun}/PLFA_{bac}$ and $PLFA_{G+}/PLFA_{G-}$, respectively. General microbial stress (*trans/cis*) was estimated as a ratio (18:1ωt + 16:1ω7t)/(18:1ω7 + 16:1ω7) [21], and nutritional stress (*cy/pre*) as (cy17:0 + cy19:0)/(16:1ω7 + 18:1ω7) [22].

2.4.2. Determination of Microbial Activities in Sediments

Activities of several specific enzymes in sediments were determined by means of spectrophotometry as the velocity of color evolution during direct incubation of the sediment sample with solutions of specific artificial substrates in Eppendorf tubes (1.5 mL). The background absorbance of a water extract of sediment was subtracted (negative control). To get closer to real environmental conditions, a reaction temperature of 25 ± 1 °C was used instead of the more common 40 °C, and the substrates were dissolved in distilled water (ambient sediment pH) instead of using buffers. Activities of phosphatases, glucosidases and proteases were determined as the velocity of p-nitrophenol evolution from p-nitrophenylphosphate (pNPP), p-nitrophenylgluco-pyranoside (pNPG) and L-alaninenitroanilide (ANA) respectively, according to an adapted method used previously [23]. Briefly, 50 mg of sediment was incubated with 0.5 mL of appropriate substrate solution. The reaction was terminated by the addition of 0.25 mL of sodium carbonate solution (1 mol/L); the mixture was centrifuged (13,000 rpm, 5 min), and the intensity of yellow color was determined at 400 nm. Absorbance of the control (sediment sample incubated in distilled water) was subtracted.

Activities of oxidases and peroxidases were determined as the velocity of oxidation of 2,6-dimethoxyphenol to pink products by oxygen or hydrogen peroxide, respectively. A portion of 50 mg of sediment was mixed with 0.75 mL of water solutions labeled control, oxidases and peroxidases. All contained 0.5 mM sodium ethylediamintetracetate; the solution for oxidases and peroxidases also contained 2,6-dimethoxyphenol (7.83 mM) and the solution for peroxidases contained hydrogen peroxide (40 μM). Activity of oxidases was subtracted from activity of peroxidases.

2.5. Determination of Nutrients in Sediments

The Mehlich 3 soil test extraction method [24] was used for the determination of plant-available main macronutrients (P, K, Ca, Mg) in soil samples with analytical determination by inductively coupled plasma optical emission spectroscopy (ICP-OES) instrumentation (namely Integra XL, GBC Scientific Equipment, Australia) according to standard operating procedures.

Sediment pH was measured in water and 0.01 mol/L $CaCl_2$ in a 1:5 sediment-to-solution ratio at 25 °C after shaking the mixture for 1 h according to ISO 10390 [25].

Oxidizable organically bound carbon C_{ox} in the sediments was determined according to ISO 14235 [26]. The sample was oxidized with an excess of potassium dichromate solution in concentrated sulfuric acid at 135 °C for 30 min. The yellow-orange dichromate ion was reduced to a green colored Cr^{3+} ion determined spectrophotometrically at 585 nm.

The content of total nitrogen (N_{tot}) in sediments was determined by digestion with concentrated sulphuric acid at 300 °C, using selenium as catalyst and salicylic acid to improve the recovery of nitrate [27], in modification of Zbíral et al. [28]. The manual steam distillation-titration method of determining ammonium nitrogen involved the liberation of ammonia with the flow of steam from the alkaline solution into boric acid indicator mixture solution. The quantity of ammonium was estimated by titration of this indicator mixture with acid.

The mineral nitrogen forms (nitrate, nitrite and ammonium) were measured in 1 mol/L of potassium chloride extract of field-moist soil samples according to ISO 14256-1 [29]. The nitrates and nitrites were determined spectrophotometrically. Nitrates present in the soil extract absorbed light radiation in the UV spectrum area (210 nm). From the assessed absorbance of the sediment extract, the absorbance of the extract after the reduction of nitrates to nitrites was subtracted. Ammonium ion determination in the sediment extract was based on the Berthelot reaction. Sodium nitroprusside was used as a catalyst for the reaction. The intensity of the blue color was spectrophotometrically assessed at 630 nm.

2.6. Statistical Analyses

Standard parametric and non-parametric statistical methods were applied to evaluate the data. Spearman's correlation coefficients were used for visitors vers., microbial and sediment nutrient data, and the Mann-Whitney test was used for pair wise comparison of sediment samples. Both the Spearman's coefficient and the Mann-Whitney test are appropriate for small samples. They are robust to outliers as well as to deviation from normal distribution of data. All correlation coefficients were tested at the 0.1 significance level. In cases of large number of samples analyzed, the one-sample Student's *t*-test was applied. To display the multivariate data, Principal Component Analysis (PCA) was applied based on the correlation matrix, and we present the so-called distance biplot. This graph shows all data points plotted in the coordinate system given by two of the components together with projection of the original axes (variables). The data points are not scaled, while the biplot eigenvectors are normalized to equal length (but not to unity, for graphical reasons). Data were analysed using Past 3.11 statistical software (Natural History Museum, University of Oslo, Norway) [30].

3. Results

3.1. Cave Attendance

The most visited cave in the interest area is the Highwayman cave (649 ± 48 persons per year), followed by the Friendship cave (535 ± 176 persons per year), and then, with less than half, the Friends of Nature cave (163 ± 68 persons per year). For other caves, values lower than 100 persons per year were recorded. During a two-year survey, 565 people completed questionnaires. The ratio of completed questionnaires corresponded to the relative attendance of individual caves and the two value sets correlated significantly (Spearman's $r = 0.95$, $p < 0.014$). This confirmed the reliability of the log-book data which were used for further calculations.

3.2. Cave and Sediment Characteristics

Significant traces of human activities were observed in caves (subjectively more abundant in caves with higher tourist traffic), especially wastes, both historical (candle residues) and recent (plastic bags, cigarettes ...). Since access to a majority of the caves is rather time consuming, activities such as eating, smoking or possibly urinating are common.

Sediment parameters are summarized in Table 2. The collected sediments varied from sandy-clay (compacted) to organic character. They were available in thin layer only and exhibited no horizontal stratification. The nutrient content of the samples was expectedly rather lower than that of common soils [14,31] and corresponded to soils on nutrient-limited substrates and soils pedogenically young. With a few exceptions, the sediment samples were quite dry (96 ± 4% dry matter) and all were acidic (pH range 3.23–6.67).

As expected, the sediment microbial characteristics indicated poor microbial communities compared to standard soils. The average concentration of $PLFA_{tot}$ (5.20 ± 0.74 mg/kg dwt) was significantly lower compared to soil collected outside near the entrances to caves (average $PLFA_{tot} = 22.10 \pm 4.79$, *t*-test, $p < 8 \times 10^{-9}$). The living microbial biomass in the caves was comparable to deserts (21.6–57.6 nmol/g PLFA ~5.5–14.7 mg/kg [32]) or young phases of soil formation by natural succession (0.17–6 mg/kg [23,33]). The content of fungal biomass (0.09 ± 0.20 mg/kg dwt, ratio $PLFA_{fun}/PLFA_{bac}$ 0.06 ± 0.08) was also low but significantly higher than 0 (*t*-test, $p < 1.8 \times 10^{-5}$). Proportions of main bacterial groups were approx. equal, i.e., 34 ± 12%, 38 ± 15% and 21 ± 13% for $PLFA_{G+}$, $PLFA_{G-}$ and $PLFA_{Ac}$, respectively. The G+/G− ratio (1.04 ± 0.57) was comparable to value of 1 (*t*-test, $p > 0.445$), common in standard soils [21]. The *trans/cis* ratio (0.2 ± 0.35), an indicator of general bacterial stress, was slightly increased from a normal value of 0.1 [21], indicating suboptimal conditions for bacterial life. The *cy/pre* ratio (1.47 ± 1.48) was significantly increased compared to the value of 0.4 (*t*-test, $p < 2.15 \times 10^{-12}$), which is considered an indicator of transition of G− bacteria to the stationary growth phase [22].

Activities of sediment enzymes were also generally low in their absolute values. Activities of phosphatases, β-glucosidases and proteases were of the order of 10^{-3} to 10^{-1} U/g dwt, comparable only to poor soils such as initial phases of primary succession on spoil heaps [23]. Nevertheless, all determined enzyme activities were significantly higher than 0 (*t*-test, highest $p < 0.002$) indicating the possibility of biological mineralization and nutrient cycling in cave sediments.

Table 2. Characteristics of determined sediment parameters and their variability.

Parameter	Avg ± Std.Dev.	Median	Lower Quartile	Upper Quartile	Min	Max	n
		Nutrients					
Dry weight	0.96 ± 0.04	0.97	0.95	0.99	0.82	1.00	45
pH (CaCl2)	4.10 ± 0.94	3.84	3.66	4.11	3.23	6.67	18
P [1] (mg/kg dwt)	264 ± 353	103	53	238	30	1241	18
K [1] (mg/kg dwt)	118 ± 148	81	50	124	15	688	18
Ca [1] (mg/kg dwt)	2151 ± 3228	270	184	1889	64	10413	18
Mg [1] (mg/kg dwt)	151 ± 159	83	67	188	31	661	18
C_{ox} (% dwt)	0.70 ± 0.41	0.75	0.32	0.94	0.09	1.53	18
humus (% dwt)	1.20 ± 0.70	1.29	0.55	1.62	0.16	2.54	18
N_{tot} (% dwt)	0.07 ± 0.05	0.06	0.03	0.1	0.01	0.23	18
$N\text{-}NH_4$ (mg/kg dwt)	28 ± 26	18	13	25	9	113	18
$N\text{-}NO_3$ (mg/kg dwt)	381 ± 520	82	11	626	4	2031	18
$N\text{-}NO_2$ (mg/kg dwt)	< LOD	-	-	-	-	-	18
		Microbial Properties (PLFA)					
$PLFA_{tot}$ (mg/kg dwt)	5.20 ± 4.74	4.51	1.82	6.64	0.37	28.16	121
F/B	0.06 ± 0.08	0.05	0	0.09	0	0.42	121
G+/G-	1.04 ± 0.57	0.89	0.66	1.34	0.00	2.92	121
Actinobacteria (%)	21 ± 13	20	12	26	1	75	121
trans/cis	0.20 ± 0.35	0.13	0.00	0.24	0	2.94	120
cy/pre	1.47 ± 1.48	1.02	0.43	1.96	0.05	8.06	120
		Enzyme Activities					
phosphatases (μU/g dwt)	30 ± 38	20	9	33	0	221	180
glucosidases (μU/g dwt)	5.4 ± 10.0	2.1	0.6	4.6	0	60.1	180
proteases (μU/g dwt)	5.3 ± 11.5	3	1.1	4.7	0	113.9	180
oxidases (nU/g dwt)	1.8 ± 3.5	0	0	2.1	0	16.3	180
peroxidases (nU/g dwt)	46.1 ± 29.5	41.0	24.4	56.5	4.3	161.1	180

[1] Extractable nutrients.

3.3. Effect of Attendance on Microbial Communities Composition

The effects of cave visitors were studied in two ways: by comparison of values for sediments compressed by trampling to non-compressed sediments (Table 3) and by correlation between the determined values and number of visitors (Table 4 shows the most important correlations, while all correlation coefficients are presented in Supplementary file Table S1a,b).

Trampling (Table 4) led to increased F/B and G+/G− ratios, increased proportion of actinobacteria in the consortium and also an increased *cy/pre* ratio. On the other hand, the activities of phosphatases, β-glucosidases and oxidases were significantly decreased in affected sediments. No significant changes were detected for values of total PLFA, *trans/cis* stress indicator, dry matter content of sediments and activities of proteases and oxidases.

Visitation correlated significantly and positively with the proportion of dry sediment matter, $PLFA_{tot}$ as well as absolute PLFA of all specific microbial groups, ratios of F/B, G+/G− and proportion of $PLFA_{Ac}$ (Table 3). It was also correlated to activity of oxidases, sediment pH, and majority of extractable mineral nutrients (P, K, Ca, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$). Significant negative correlations were observed for stress indicator *trans/cis*, and activities of phosphatases and peroxidases. No significant correlation was observed for activities of glucosidases, proteases, extractable Mg, N_{tot}, C_{ox} and the humus content.

Table 3. Comparison of trampling effect on sediment characteristics (Mann-Whitney test). Significant differences ($p < 0.1$) are indicated in bold.

Variable	Median (Trampling = 0)	Median (Trampling = 1)	*p*-Value
$PLFA_{tot}$ (mg/kg dwt)	4.67	4.41	0.928
$PLFA_{fun}$ (mg/kg dwt)	**0**	**0.06**	**2.0×10^{-10}**
$PLFA_{bac}$ (mg/kg dwt)	**0.58**	**1.52**	**6.5×10^{-4}**
$PLFA_{G+}$ (mg/kg dwt)	**0.2**	**0.54**	**7.6×10^{-7}**
$PLFA_{G-}$ (mg/kg dwt)	**0.19**	**0.475**	**4.2×10^{-3}**
$PLFA_{Ac}$ (mg/kg dwt)	**0.08**	**0.31**	**2.02×10^{-5}**
F/B	**0**	**0.065**	**2.86×10^{-5}**
G+/G-	**0.71**	**1.185**	**5.3×10^{-5}**
trans/cis	0.12	0.14	0.485
cy/pre	**0.54**	**1.365**	**9.3×10^{-7}**
Dry weight	0.95	0.96	0.418
Phosphatases (U/g d.wt)	**0.029**	**0.017**	**0.0112**
β-glucosidases (U/g d.wt)	**0.004**	**0.0013**	**0.0112**
Proteases (U/g d.wt)	0.0032	0.0031	0.933
Oxidases (U/g d.wt)	0	9.1×10^{-8}	0.376
Peroxidases (U/g d.wt)	**4.6×10^{-5}**	**3.5×10^{-5}**	**0.025**

Table 4. Main Spearman correlations between parameters (complete tables are available in Supplementary Material Table S1). Significant correlations ($p < 0.1$) are indicated in bold.

Parameter	Attendance	$PLFA_{tot}$
$PLFA_{tot}$ (mg/kg dwt)	**0.57**	-
$PLFA_{fun}$ (mg/kg dwt)	**0.60**	**0.38**
$PLFA_{bac}$ (mg/kg dwt)	**0.46**	**0.81**
$PLFA_{G+}$ (mg/kg dwt)	**0.47**	**0.71**
$PLFA_{G-}$ (mg/kg dwt)	**0.28**	**0.74**
$PLFA_{Ac}$ (mg/kg dwt)	**0.52**	**0.72**
F/B	**0.54**	**0.17**
G+/G−	**0.40**	−0.03
Actinobacteria (%)	**0.34**	**0.16**
trans/cis	**−0.46**	**−0.40**
cy/pre	−0.13	**−0.20**
phosphatases (μU/g dwt)	**−0.26**	−0.11
glucosidases (μU/g dwt)	−0.18	−0.07
proteases (μU/g dwt)	0.16	0.03
oxidases (nU/g dwt)	**0.32**	0.06
peroxidases (nU/g dwt)	**−0.68**	−0.33
Dry weight	**0.75**	**0.75**
pH ($CaCl_2$)	**0.60**	−0.12
Extractable P (mg/kg dwt)	**0.76**	0.32
Extractable K (mg/kg dwt)	**0.83**	0.23
Extractable Ca (mg/kg dwt)	**0.83**	**0.75**
Extractable Mg (mg/kg dwt)	0.38	0.00
C_{ox} (% dwt)	−0.38	−0.39
humus (% dwt)	−0.38	−0.39
N_{tot} (% dwt)	0.47	0.10
$N\text{-}NH_4$ (mg/kg dwt)	**0.60**	−0.12
$N\text{-}NO_3$ (mg/kg dwt)	**0.83**	0.23

4. Discussion

The results clearly indicate the expected effect of visitors on sediment microbial communities. Sediment and microbial characteristics significantly differed between soil affected and not affected by trampling, and they were also significantly correlated to number of visitors.

From a microbial point of view, the effect is positive. More visitors attending the caves resulted in higher indicators of total microbial biomass and increases of biomass for all microbial groups. The relative increase of biomass of G− bacterial was lower compared to other microbial groups, resulting in a proportional decrease of this group within the bacterial community and an increased G+/G− ratio. Significant correlation of visitors with the F/B ratio indicates a more positive effect of visitors on fungi than on bacteria. Of significant importance is the negative correlation of number of visitors with both stress indicators, i.e., visitors contributed to improved microbial physiology. Analogous effects were observed for sediments affected by trampling, which also led to an increase in the majority of microbial parameters. The only exception was the *cy/pre* stress indicator, where the trampling led oppositely to a significant increase. Since in general this is an indicator of transition of G− bacteria to stationary growth phase [21,22], this could be attributed to spatial limitation in compacted sediments.

Two hypotheses were formulated to explain the mechanism of the effect of visitors on microbial communities:

1. increased input of nutrients caused by visitors,
2. input of external microorganisms.

The first hypothesis of nutrient input into caves is a very likely mechanism, since visitors often leave waste in the caves, eat, drink, smoke, and even urinating is possible. Nutrients can also be imported on shoes. Other authors also consider this mechanism important [8]. This hypothesis is supported mainly by correlation of the majority of available nutrients (P, K, Ca, $N\text{-}NO_3^-$, $N\text{-}NH_4^+$) with number of visitors (Table 4). In addition, the determined enzyme activities predominantly decreased with number of visitors. Higher activity of extracellular hydrolytic enzymes is believed to indicate increased effort of microorganisms in obtaining nutrients [34,35] and thus the decrease supports the nutrient input hypothesis. The effect is even more pronounced if the activities of extracellular enzymes are expressed specifically per unit of biomass (not shown).

The second hypothesis, i.e., the input of external microorganisms into caves, was also proposed by other researchers [7,36]. Our results support this hypothesis based on significantly richer microbial community in sediments compacted by trampling. The presence of pieces of richer soil from outside on visitors' shoes is likely. Nevertheless, the PLFA analysis does not provide sufficiently detailed data to confirm the similarity of microbial patterns of outside soil and trampled sediments, nor it is suitable [37]. Different methods, such as metagenomics, would be required to finally confirm or accept this hypothesis.

Overall, we can conclude that it is likely that both mechanisms play a role in the attendance effect.

4.1. Implications for Regulation

Previous research on cave microbial communities indicates that caves serve as reservoirs of unique and non-described species. Protection of these ecosystems is therefore of interest for biodiversity preservation [7,36]. Although the effect of visitors on microbial communities overall is positive, from a nature conservation point of view, where unaffected and natural ecosystems should be protected, the observed effect is negative. Tourist traffic represents a significant influence on natural ecosystems; moreover, in a highly protected nature area (National Nature Reservation—NNR) (In the legislative of the Czech Republic "National Nature Reservation" presents a very strict form of natural protection and is defined by Act no. 114/1992 Coll., on Nature and landscape protection as amended (in Czech "Zákon ČNR č. 114/1992 Sb., o ochraně přírody a krajiny, v platném znění")). Even though the highest attendance determined in the area is two orders of magnitude lower compared to caves serving as a touristic magnet ([8]—200,000 visitors annually), we propose taking measures to regulate the number of allowed visitors in the caves. The key question is the acceptable number of visitors. Unlike other studies comparing visited and restricted caves [7], in this area there was no similar cave with zero attendance available for comparison. Therefore, the recommendation can only be derived based on comparison of low-traffic caves and high-traffic caves. To highlight the visitors' effect, we have therefore focused our attention on samples most affected by visitor activity, i.e., to samples compacted by trampling (ignoring non-trampled samples). The samples were divided into three categories based on the annual number of visitors in the caves (low $\leq$ 30, medium 31–499, high $\geq$ 500); an approach similar to that described by Ikner et al. [8]. These three groups present a compromise resulting from the number of analyzed caves and distribution of their annual attendance values. Principal component analyses of PLFA data (Figure 2) revealed a similar pattern of data variability for low and medium traffic caves in contrast to different patterns of high traffic caves. If we assume the effect of low attendance as acceptable, we can therefore accept the medium attendance as acceptable. This comparison indicates that the possible acceptable limit of visitors could lie in the "medium" range, i.e., below its upper limit of 165 annual visitors. The results will be presented to local authorities and management of the Protected Landscape Area Labské Pískovce.

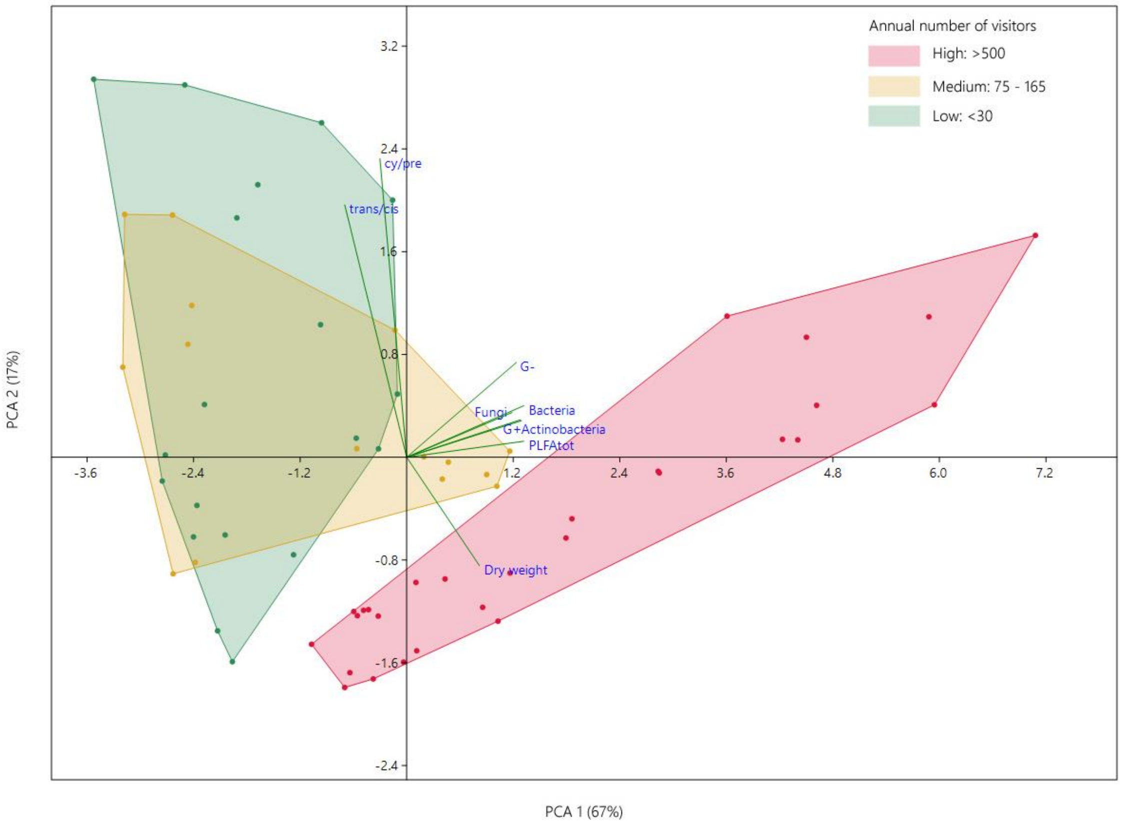

Figure 2. Principal component analyses of PLFA values from samples affected by trampling.

5. Conclusions

Sediment microbial communities in 12 sand-stone caves in PLA Labské Pískovce (Czech Republic) were studied by PLFA analyses and determination of extracellular enzyme activities. We found a significant increase of certain microbial characteristics with annual number of visitors, which indicates significant changes of some other components of cave ecosystems by visitors, most likely by sediment eutrophication. Since the access to these caves is upon permission only, we therefore propose regulation of visits to these caves and limit them to an acceptable number not exceeding 165 visitors annually.

Supplementary Materials: The following are available online at www.mdpi.com/2071-1050/10/2/396/s1, Figure S1: Example of questionnaire form distributed in caves, Table S1a: All Spearman correlations between variables (based on pair values), Table S1b: Spearman correlations between enzyme activities and other parameters (based on average values).

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Author Contributions: Jaroslav Kukla and Michal Holec initiated the research; Jaroslav Kukla carried out the sampling, attendance analyses and PLFA analysis; Michal Holec contributed to sampling and relation of results to ecological parameters and contributed to manuscript preparation; Josef Trögl managed the microbiological analyses, contributed to data evaluation and preparation of the manuscript; Diana Holcová contributed to attendance evaluation and preparation of the manuscript; Dagmar Hofmanová. carried out the analyses and evaluation of enzyme activities; Pavel Kuráň carried out GC-MS analyses of fatty acid methyl esters; Jan Popelka carried out the statistical evaluation of the data; Jan Pacina evaluated the GIS data and prepared the maps; Sylvie Kříženecká, Sergej Usťak and Roman Honzík carried out the supplementary sediment analyses.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A. —Brief History of Cave Visiting in the Area

Some of the caves were probably known already to the first people in the area. Some of the caves appear in the maps from the 16th century, especially due to close land borders. Some of the caves were probably visited at those times (e.g., the most visited Loupežnická jeskyně = Highwayman cave). Also, from this cave, the oldest log-book has been preserved covering the time period 1912–1928. Nevertheless, the entire chronosequences up to date have not been preserved in majority of cases. Visiting of the caves started to be more frequent at the turn of 19th and 20th centuries as a consequence of industrial revolution and increasing interest in nature. The oldest dated record from 1881 was found in Stelzig cave. At the beginning of the 20th century, the systematic speleological mapping of the area was carried out, especially by German people both from Czechoslovakia and across the border, i.e., Saxony. Also, the first information on organized guided tourism dates to this time [38].

Between 1945 and 1948, as a consequence of the World War II, majority of the German nationality population (approx. 3 milion people) was expulsed from Czechoslovakia [39]. In the area of interest, this meant more than 50% (often up to 100% of citizens). With population exchange, the knowledge of caves temporarily disappeared.

The last important milestone in the cave visiting history was the fall of the communist regime in Czechoslovakia and Eastern Germany in 1989. Since then, many activities have been developing, both touristic (including e.g., popular geocaching) as well as research. Twenty-two new caves were discovered; in others, new spaces were mapped. This also led to an increase interest in cave protection and research of unique ecosystems.

Appendix B. –Demographic Data of Cave Visitors

The demographic data showed that men respondents dominated over women (74.16 vs. 25.84%). The largest number of respondents belonged to the age category of 25–39 years (42.65%), followed by 21.77% to the category of 18–24 years, 20.35% of 40–59 years, 14.87% of 0–17 years and 0.35% to the category of 60 and more years. Further, respondents with secondary education constituted 45.31%, followed by university (43.54%) and elementary (11.15%) educated respondents. The German and Czech nationality were the majority of respondents (64.07 and 33.98%). A total of 15.41% of respondents come from a distance within 20 km, 12.72% from a distance of 20–40 km, 34.68% of 40–80 km, 15.22% of 80–160 km, 14.45% of 160– 320 km and 7.51% of 320–640 km. The largest number of respondents came from the Dresden region (33.27 %), and the Ústecký region (15.04%). Further, based on distance: Berlin (6.55%), Chemnitz (5.84%), the Liberec region (5.66%), Praha (4.78%), Leipzig (3.54%), Thüringen (2.65%), Schleswig-Holstein (2.30%), the Central-Bohemian region (2.3%), and Brandenburg (1.95%). People were most often transported to the vicinity of the caves by car (78.58%), followed by train (14.51%), bus (4.96%) or otherwise (1.95%). Most respondents spent more than 3 h (24.42%) or less than 1 h (23.01%) on the way to the visited cave. Then, times of within 1.5 h 17.35%, within 2 h 13.98%, within 3 h 12.57% and within 0.5 h 8.67% followed. Most respondents heard about caves from a second person (67.79%), or from literature (15.58%), internet (8.85%) or otherwise (7.79%). Most respondents visited a cave without a professional guide (61.95%). The number of respondents who used the services of a professional guide (38.05%) varied considerably between individual caves. Most respondents visited the caves of PLA Labské pískovce for the first time (58.58%); a smaller portion visited them occasionally (29.91%), and the lowest number of respondents regularly (11.50%). Respondents were the most interested in climbing (44.25%), other interests (27.43%), tourism (20.71%), geocaching (4.07%) and speleology (3.54%). Respondents were encouraged to visit the cave by adrenaline and adventure (50.62%), sport activity (30.27%), other motivation (7.43%) and interest in science and natural knowledge (6.55%); the cave visit was the main aim for most of them (73.27%). Most respondents intended to visit the caves again (60.18%).

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