Microb Ecol (2016) 72:163–174
DOI 10.1007/s00248-016-0760-6

ENVIRONMENTAL MICROBIOLOGY

Substantial Variability of Multiple Microbial Communities Collected at Similar Acidic Mine Water Outlets

Lukáš Falteisek[1] · **Vojtěch Duchoslav**[2] · **Ivan Čepička**[1]

Received: 24 July 2015 / Accepted: 28 March 2016 / Published online: 8 April 2016

Abstract Deep sequencing of prokaryotic 16S rDNA regularly reveals thousands of microbial species thriving in many common habitats. It is still unknown how this huge microbial diversity, including many potentially competing organisms, may persist at a single site. One of plausible hypotheses is that a large number of spatially separated microcommunities exist within each complex habitat. Smaller subset of the species may exist in each microcommunity and actually interact with each other. We sampled two groups of microbial stalactites growing at a single acidic mine drainage outlet as a model of multiplicated, low-complexity microhabitat. Samples from six other sites were added for comparison. Both tRFLP and 16S rDNA pyrosequencing showed that microbial communities containing 6 to 51 species-level operational taxonomic units (OTU) inhabited all stalactites. Interestingly, most OTUs including the highly abundant ones unpredictably alternated regardless of physical and environmental distance of the stalactites. As a result, the communities clustered independently on sample site and other variables when using both phylogenetic dissimilarity and OTU abundance metrics. Interestingly, artificial communities generated by pooling the biota of several adjacent stalactites together clustered by the locality more strongly than when the stalactites were analyzed separately. The most probable interpretation is that each stalactite contains likely random selection from the pool of plausible species. Such degree of stochasticity in assembly of extremophilic microbial communities is significantly greater than commonly proposed and requires caution when interpreting microbial diversity.

Keywords Acidophiles · Chemolithotrophy · Phylogenetic dissimilarity · Community assembly · Neutral processes

Introduction

Analyses of microbial communities from ecological and biogeographical points of view have recently undergone a rapid progress, thanks to modern sequencing methods. Despite this progress, we are still far from assessing the contribution of environmental filtering and other processes, such as interspecific interactions, dispersal limitation, and colonization history to the microbial community structure [1, 2]. One of so far unexplained phenomena observed in all but most extreme environments is the high level of prokaryotic diversity that is considered hardly sustainable by the theory of ecological communities [1]. Additionally, Nemergut et al. [3] showed that multiple deeply sequenced samples from similar environments show markedly low overlap of prokaryotic 16S rDNA genotype composition. This finding was reproduced in various types of environments on a wide scale of geographical distances and operational taxonomic unit (OTU) definitions [3]. The main problem in understanding the mechanisms that sustain the large diversity is our inability to scale down the sampling in order to examine the true habitats of microbes. Thus, it cannot be determined whether ecologically similar organisms detected within a single sample inhabit spatially isolated microhabitats or are equally distributed. The

Electronic supplementary material The online version of this article (doi:10.1007/s00248-016-0760-6) contains supplementary material, which is available to authorized users.

✉ Lukáš Falteisek
nealkoholik@email.cz

[1] Department of Zoology, Faculty of Science, Charles University in Prague, Viničná 7, 128 44 Prague, Czech Republic

[2] Department of Ecology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

important difference between the abovementioned scenarios is that direct competition is enabled only in the latter case [1].

Extreme habitats dominated by bacteria, such as chemolithotrophic microbial growths in acidic or sulfidic waters, host conspicuously low microbial diversity compared to common environments. Only a few species occupying key ecological roles are usually detected at the extreme sites [4–7]. Distinct partitioning of niches among competing microbes with slightly differing growth optima has been reported in microbial communities developing in habitats with low complexity in extreme environments [8, 9].

Diversity of microbial species and metabolisms at various sites impacted by acidic mine drainage (AMD) generated by dissolution of sulfide minerals is described better than in common, highly diversified environments, including soils or surface waters [10]. Many prokaryotic species typically recovered from AMD environments were isolated in culture and formally described [11–15]. However, the majority of the studies were descriptive rather than attempting to get a deeper insight into the ecological processes. Studies focusing on microbial diversity typically employed deep sequencing of a few samples [16] or pooled drip-water from a larger seepage area [17] in order to characterize the typical composition of the microbial assemblages, and thus did not take into consideration distribution of particular species to individual microhabitats. Data from other environments suggest that bacteria can be non-equally distributed among related microhabitats. It has been shown that physiologically similar but genetically distinct populations of bacteria alternate in both spatial and temporal manners in lake biofilms [18].

Typical examples of well-described AMD-related extreme habitats are outlets of iron-rich, acidic mine water from rock fissures [17, 19, 20]. These outlets commonly host macroscopic growths of prokaryotic microorganisms forming stalactite-like structures (i.e., snottites) composed of bacterial biomass and secondary minerals. The species composition of the stalactites is similar to the communities found at other habitats associated with AMD flow. The most conspicuous example are gelatinous snottites strikingly dominated by *Ferrovum myxofaciens* and *Acidithiobacillus ferrivorans*, which are known from both AMD streams and stalactites [4, 15].

The thorough description of organisms inhabiting AMD-related microbial stalactites encourages employing them as a model for research on the variability of natural microbial communities. It has been shown that the environment at the terminal part of a stalactite is relatively uniform despite a slight gradient of oxygenation and iron redox state along the stalactite body [19]. At least, the terminal part of the stalactite represents a homogenous habitat that hosts microbial community with limited diversity. Additionally, the stalactites grow commonly in large groups at a single water seepage area. Stalactites in each such group, supplemented by the same AMD source (i.e., one permeable section of a rock fracture), represent multiplied microhabitats of a single type. The local environments of each microhabitat show the highest possible rate of similarity that is accessible in naturally occurring microbial habitats. Yet, these microenvironments are physically isolated from each other, and thus, each of them has an individual history. Therefore, we should be able to observe the residual heterogeneity of microbial communities by comparing individual stalactites, while the influence of environmental variability is suppressed.

In this study, we reveal microbial diversity of 51 stalactites and snottites representing two groups, each collected from a single AMD outlet, and ten similar samples from variably distant sites, both geographically and environmentally. We employed tRFLP and 16S rDNA pyrosequencing to compare composition of microbial communities intentionally collected at maximally similar adjacent sites with communities from additional more varied sites in four abandoned mines.

Materials and Methods

Site Description

The samples were collected in four abandoned sulfidic ore mines. Three of them are located in the Czech Republic (Zlaté Hory, Lehnschafter, and Kristýnov) and one in Slovakia (Banská Štiavnica).

The Zlaté Hory-south deposit was described in Falteisek and Čepička [20]. Briefly, it is located in Silesia (Czech Republic; 50°12′N, 17°24′E) and represents a large Rosebery type volcanosedimentary copper deposit [21]. The pyrite-chalcopyrite ore is dispersed in metamorphic Devonian quartzites and shales, forming extensive ore bodies. Mining activities ceased in 1990. The extensive supergenic processes generating acidic mine drainage (AMD), accompanied by typical secondary zonation, were described by Martyčák et al. [22]. All samples were collected at or close to the 3rd haulage level, which represented the typical oxidation zone (190–220 m below the surface).

The Lehnschafter mine in the town of Mikulov is located in Ore Mountains in north-western Bohemia (50°41.46′N, 13°43.28′E). It represents a vein-type Pb-Ag-As sulfidic deposit with orthogneiss and rhyolite as adjoining rocks. The mining operations lasted from the late Middle Ages to 1885. The AMD-related environments are developed locally throughout the mine and depend on weathering of pyrite and arsenopyrite.

Kristýnov mine is located near Plzeň city in western Bohemia (49°43.61′N, 13°26.2′E). The deposit is located in upper proterozoic pyritic shales and was partially exploited by pillar mining in the nineteenth century. The mine is shallow (ca. 15 m) and almost dry, with only local weak seepages of AMD.

Banská Štiavnica mine district is located in Štiavnica Mountains, Slovakia (48°27′N, 18°53′E). It is a large polymetallic deposit consisting of veins and impregnations of Fe, Cu, Pb, Zn, and Ag sulfides developed mostly in andesites and quartz-diorite porphyry. The mining operations ceased in 1993. The AMD seepages occur at multiple sites where significant pyrite impregnations exist. The sampling sites are located in modern mining galleries at the 12th haulage level, ca. 550–600 m below the surface.

Sample Collection

The stalactites for microbiological analyses were harvested directly into sterile 15-mL tubes. Information about their physical appearance (hard, gelatinous, encrusted) and approximate water flow was collected as well. Conductivity, pH, and temperature of the water for samples with significant water flow were measured using WTW Multi303i universal pocket meter (WTW, Germany). Water samples for chemical analyses were collected into clean 50-mL tubes. The water flow was estimated from the filling times of the sample tubes (the flow was directed into the tube quantitatively). Most samples were delivered to the laboratory within 5 h; the samples from Banská Štiavnica within 12 h. After transport, the water was filtered through 0.22-μm filter with PES membrane (Carl Roth, Germany), and the aliquots designated for analysis of cations were stabilized by 2 % HNO_3 (suprapure, Merck). The solid samples were stored in −80 °C until the DNA extraction.

Chemical Analyses of Water

Anions (SO_4^{2-}, F^-, Cl^-, Br^-, PO_4^{3-}, and NO_3^-) were analyzed by ion chromatography (HPLC, column Dionex ICS-2000), with a precision of about ±8 %. Major and minor cations (Fe, Al, Cu, As, Si, Ca, Mg, and Mn) were analyzed by ICP OES analyzer iCAP6500 (Winsford, UK).

DNA Extraction, Amplification, and tRFLP Analyses

The stalactites were divided by sterile lancet and forceps. One to three sections ca. 1-cm long were dissected from the near-end region of each sample and used for DNA extraction. Genomic DNA was isolated using ZR soil microbe DNA kit (Zymo research). The universal primers U515F (GTGCCAGCMGCCGCGGTAA) and U1406R (GACGGGCGGTGTGTRCA) [23] at annealing temperature of 55 °C and LA DNA polymerase (Top-bio, Czech Republic) were used to amplify approximately 860-bp long fragment containing V4–V8 regions of the 16S rDNA from *Bacteria*, *Archaea*, and mitochondria.

Primers U515F-A488 and U1406R-A546 labeled by AlexaFluor dyes were used for PCR prior to tRFLP. The PCR products were purified by agarose electrophoresis, digested by HaeIII (Thermo Scientific), and analyzed by 3130 Genetic Analyzer (Applied Biosystems).

454 Pyrosequencing and Sequence Analyses

PCR amplification of 16S rDNA was performed in triplicates as described above. Products were purified using Zymoclean™ Gel DNA recovery kit (Zymo Research), mixed, and subjected to short (10-cycle) secondary PCR with primers containing 6-bp sequence tags unique for each sample. The tagged PCR products were mixed equally and sequenced on a GS FLX Titanium Platform (Roche) at the Laboratory of Environmental Microbiology, Institute of Microbiology of the ASCR, Prague, Czech Republic.

Sequence data were analyzed by the Seed pipeline [24]. Sequences were extracted and denoised using Mothur 1.28.0 [25]. The reads from both ends of the amplified fragment were present due to random ligation of pyrosequencing adaptors. Only forward reads (i.e., from primer U515F) were selected for further analyses. They were clustered into OTUs with 97 % threshold by usearch 5.2.32 [26], and chimeras were removed by uclust 3.0.167 [26]. Species identities of the consensus sequences were assessed by BLASTn 2.2.26+ [27] against NCBI nt/nr database. Singletons were excluded from further analyses due to a high number of suspect erroneous sequences.

The consensus sequences were aligned using MAFFT [28] with help of the CBRC server (http://mafft.cbrc.jp/alignment/server/). The alignment was manually edited, and a maximum-likelihood phylogenetic tree was constructed by Phyml 3.0 [29] under GTR + Γ + I substitution model.

The newly determined sequences have been deposited in MG-RAST [30] under project ID 12174, and selected representatives of species-level OTU from each sample were deposited in GenBank under accession numbers KP688592–KP689090.

Statistical Methods

Multivariate analyses of tRFLP patterns were run in Canoco 5.0 [31]. The variability within the dataset was visualized using a detrended correspondence analysis (DCA), and canonical correspondence analysis (CCA) was applied to test whether the differences in tRFLP patterns are significantly related to the stalactite properties and sampling sites. Localities were used as explanatory variables and the other properties (dripping rate, encrustation, and physical character) as covariates. We created dummy variables for each of these variables. The dripping rate was transformed into semi-quantitative categories for this purpose. Association of terminal restriction fragments (i.e., normalized peak area) with sampling sites was tested using one-way ANOVA for each fragment separately. Localities were used as categorical factors and peak areas as

dependent variables. The association of a fragment with sample sites was assessed from F value and p value of the ANOVA.

Clustering of the microbial communities based on a set of if-then logical split conditions was determined from the presence/absence and abundance of principal OTUs using tree-building algorithms in Statistica software package [32]. All samples were randomly subsampled to 300 sequences for this analysis.

The phylogenetic dissimilarity of microbial communities, principal component analyses (PCoA), and UPGMA clustering were performed in Fast UniFrac software package [33] using the maximum-likelihood phylogenetic tree of consensus sequences of individual OTUs. Both weighted normalized and unweighted algorithms were employed. For Jackknife analysis, all the samples were transformed to 1000 sequences per sample, and 75 % of sequences were kept in each replicate.

Results

Field Observations and Sampling

We sampled 51 ferruginous stalactites from eight sites located in four abandoned underground mines (Table 1). The four sites were in Zlaté Hory-south mine (ZH). The ZH-A is a group of gelatinous and hard soda-straw stalactites that grow from water seepage from two parallel rock bedding planes in an area of ca. 0.2 × 1 m. The water composition was considered to be homogeneous within the site due to the small area and existence of abundant fractures that served as cross-conduits at a centimeter scale (Fig. S1a). The group ZH-B consisted of hard soda-straw stalactites growing from a hard ferrous crust that covered the roof and walls of the mine gallery. Only the central part (ca. 1-m wide) of the large group of stalactites was sampled. Samples ZH7 and ZH10 represent massive, partially gelatinous stalactites growing on greater springs. Both sampling sites were described in detail by Falteisek and Čepička [20]. All the sites in Zlaté Hory mine were localized around a single ore body with the maximum distance between each other being 100 m. Samples from Kristýnov mine (KU) represent six hard or gelatinous stalactites from a single weak seepage area ca. 30-cm wide. In the Lehnschafter mine (MI), a gelatinous snottite flourishing at a water seepage from caved mine gallery was sampled. The main difference from the other sites was a high content of arsenic in the water (Table 1) causing precipitation of amorphous ferric arsenate (pitticite) inside the gelatinous material. Banská Štiavnica mine (BS, BN) differed from the others in temperature, which reached 20–24 °C, in contrast to 9–10 °C in the other mines. Sample BS was an acidic, gelatinous gypsum-encrusted stalactite growing on extremely mineralized, As-containing water. Stalactite BN was uniquely found at AMD seepage neutralized by filtration through the concrete support of the mine gallery. The stalactite consisted of mineralized and gelatinous parts that were obviously different and were processed as two samples.

tRFLP Analyses

Characteristic patterns with a few dominant peaks, indicating highly unequal abundance of 16S rDNA genotypes (i.e., single or a few highly dominant species in each community), were obtained for all the samples. DCA analysis was performed in order to preliminarily classify tRFLP patterns. The samples formed several visually distinct clusters and gradients (Fig. 1a). Samples selected for subsequent pyrosequencing were dispersed evenly on the DCA plot and thus probably represented the true scale of the microbial community types.

To evaluate possible association of the terminal restriction fragments (TRFs) and sample sites, CCA analyses of all the TRFs, as well as selected TRFs, which represented more than 10 and 20 % of total peak area at least in one sample, were performed. The log-transformed peak areas were used in all the analyses. The sample sites were used as explanatory variables, and the other stalactite properties were included as covariates. Variability explained by sampling site was 30.8 % ($p = 0.002$) for all peaks (Fig. 1b), 27.6 % ($p = 0.002$) for peaks over 10 % of the total peak area (Fig. 1c), and 22.2 % ($p = 0.02$) for peaks over 20 % of the total peak area (data not shown). Although certain localities appeared divergent in the CCA analyses, the huge majority of peaks clustered in the center with no clear association with localities. Significance of association of peaks and localities was tested by ANOVA for each single peak. This analysis showed that most of TRFs, except for several, mostly small, peaks, were not associated with sample site (p value median = 0.22). Additionally, the TRFs corresponding to most abundant species-level OTUs as determined by 16S rDNA sequencing were identified and analyzed in the same way. The TRF of *Thiobacillus aquaesulis* was found only in samples from site BN; the rest of the identified TRFs revealed only a weak association with sampling sites (Fig. 1d, ANOVA p value median = 0.09). No correlation between TRF's total or average area and the strength of association with sampling site was detected indicating that more abundant organisms were neither more generally distributed nor more confined to certain sites ($p = 0.6$). The exception of *T. aquaesulis* may be easily explained by the different chemism of the BN site (see above).

16S rDNA Pyrosequencing

A subset of 24 samples from 21 stalactites was subjected to pyrosequencing of a fragment of 16S rDNA. Samples from two stalactites were doubled to test homogeneity of the microbial assembly, and both hard encrusted and snotite-like

Table 1 Number of samples collected at the sampling sites, characterization of the sites, and water that flowed through the stalactites

Locality		Zlaté Hory				Kristýnov	Lehnschafter	Banská Štiavnica		Total
Sampling site		ZH-A	ZH-B	ZH7	ZH10	KU	MI	BS	BN	
No. stalactites		21	19	1	1	6	1	1	1	51
tRFLP samples		23	22	1	1	6	1	2	2	58
Sequenced		9 (7 stal.)	8	1	1	1	1	1	2 (1 stal.)	24 (21 stal.)
Temperature	°C	9.5	9.5	9.5	9.5	10	9	22	20	
Water flow	L/s	low	low	0.04	0.2	0	0.02	0.01	0.02	
pH		ca. 3	ca. 3	2.95	3.6	ca. 3	2.68	1.92	7.07	
Conductivity	mS/cm	n.a.	n.a.	4.8	1.86	n.a.	2.3	30.8	n.a.	
Fe	mg/L	n.a.	n.a.	774[a]	n.a.	n.a.	272	18,780	2.4	
Al	mg/L	n.a.	n.a.	131[a]	n.a.	n.a.	22	510	0	
Cu	mg/L	n.a.	n.a.	90[a]	n.a.	n.a.	0.1	1130	0.0011	
As	mg/L	n.a.	n.a.	0.225[a]	n.a.	n.a.	4.99	50	0.00011	
Si	mg/L	n.a.	n.a.	18[a]	n.a.	n.a.	4.05	110	6.48	
Ca	mg/L	n.a.	n.a.	49.7[a]	n.a.	n.a.	112	500	205.8	
Mg	mg/L	n.a.	n.a.	97.4[a]	n.a.	n.a.	26.3	710	47.4	
Mn	mg/L	n.a.	n.a.	26.7[a]	n.a.	n.a.	3.22	1180	0.05	
SO_4^{2-}	mg/L	n.a.	n.a.	3509[a]	n.a.	n.a.	943	42,314	466.65	
Cl^-	mg/L	n.a.	n.a.	8.3[a]	n.a.	n.a.	2.45	<1	3.64	
NO_3^-	mg/L	n.a.	n.a.	2.7[a]	n.a.	n.a.	<0.2	<2	11.23	

[a] Water composition of ZH7 after Falteisek and Čepička [19]

sections were sampled from the stalactite BN. We obtained 78,077 reads longer than 200 bp in two orientations; 31158 quality-filtered, non-chimeric forward reads were used for further analysis. Microbial diversity ranged from 6 to 51 OTUs per sample at 97 % similarity level (median 28). When sorted by frequency, the top 80 % of sequences represented from 1 to 14 OTUs (median 5). The Chao-1-estimated richness [34] varied from 6 to 53 OTUs per sample (median 36.5; Table S1). In total, 187 OTUs at 97 % similarity level were defined (Table S2). Two OTUs with 42 sequences belonged to *Archaea*, 10 OTUs with 74 sequences were eukaryotic nuclear 18S rDNA sequences, and 10 OTUs with 409 sequences were identified as mitochondrial 12S rDNA. The archaeal and mitochondrial sequences were included in the dataset. Eukaryotic nuclear sequences were discarded due to their heterogeneity and probable bias in the recovered eukaryotic community composition. When all the samples were equalized to 300 randomly selected sequences per sample, a highly similar distribution of 162 OTUs was revealed.

The OTUs related to a known organism with sequence homology greater than 98 % were assigned to this species. Abundant OTUs with lower homology were assigned to higher-level taxa (genera, families, etc.). The most abundant organisms in most samples were iron-oxidizing chemoautotrophs (*F. myxofaciens*, *A. ferrivorans*, *Gallionella capsiferriformans*, *Sideroxydans litotrophicus*, *Acidiferrobacter thiooxidans*). Seven samples lacked autotrophs completely or almost completely and were dominated by acidophilic heterotrophic bacteria (*Acidiphilium sp*. PK48, *Acidocella* sp. CFR23, *Metallibacterium* sp. X11) and even organisms closely related to ubiquitous neutrophilic heterotrophs (*Burkholderia* sp., *Bradyrhizobium elkanii*, *Flavobacteria* gen. sp.) (Table 2). Only 14 OTUs reached more than 20 % of sequences in at least one sample. They seemed to form conserved or semi-conserved associations that alternated in the stalactites and usually were not confined to a single sample site. To test this suggestion, an analysis of microbial communities by weighted normalized Fast UniFrac using maximum-likelihood phylogenetic tree of consensus sequences was performed. It revealed four clusters (C1–4) of microbial communities with high statistical support in the jackknife analysis (Fig. 2a, b). The four samples were associated with these clusters but with low statistical support, or remained unclustered. Duplicates from a single stalactite revealed similar composition in both cases. A similar clustering was also obtained by a non-phylogenetic approach based on tree-building algorithm (Fig. S2a). Interestingly, all the clusters but C3 contained stalactites found at more sites, and communities belonging to various clusters were found in stalactites from both deeply sampled sites ZH-A and ZH-B, showing that the clusters were not site-specific. The communities of cluster C2, which was strikingly dominated by *F. myxofaciens*,

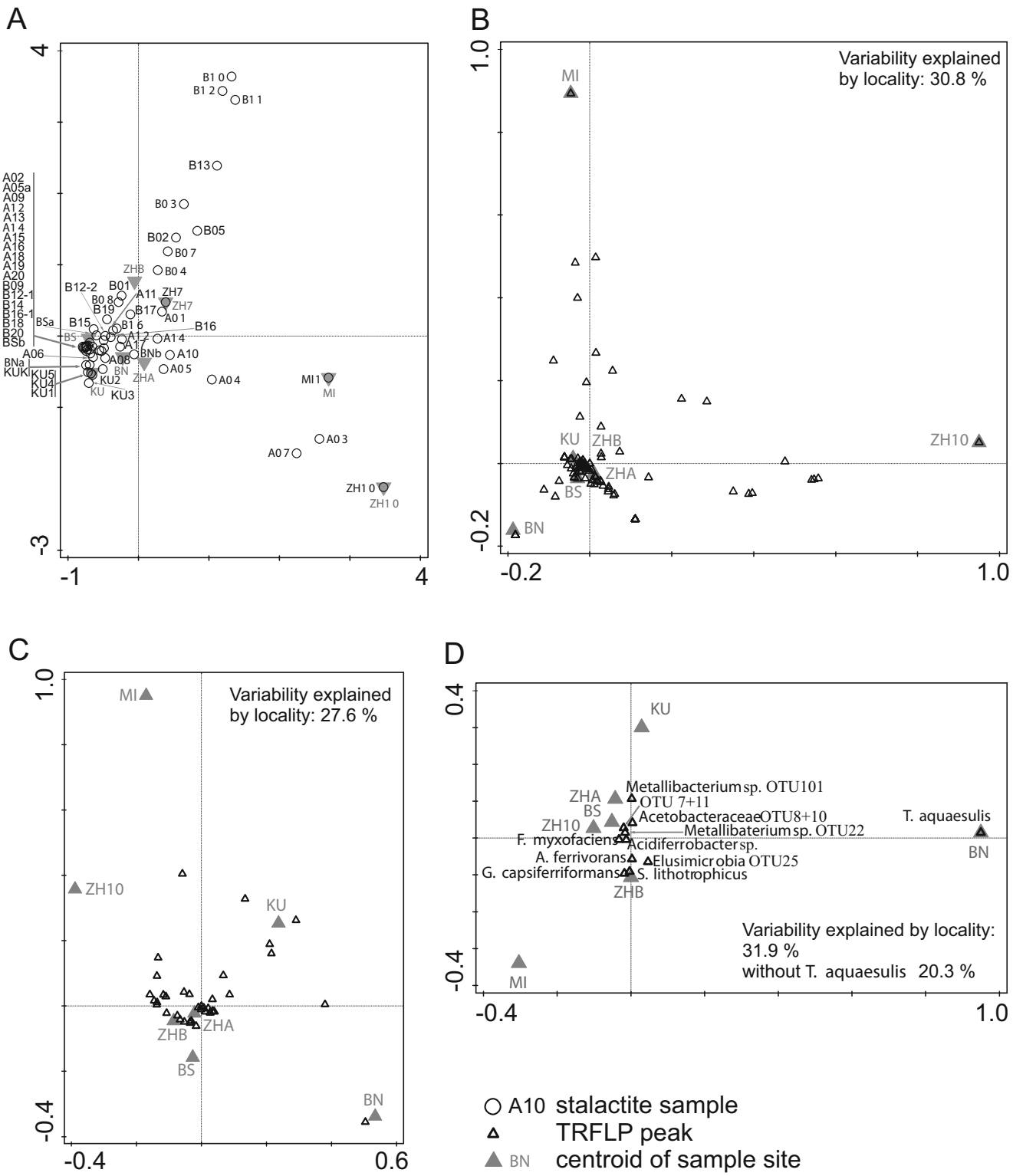

Fig. 1 Results of multidimensional analyses of 16S rDNA tRFLP profiles of 58 samples representing 52 stalactites. The analysis was performed using log-transformed peak areas. Replicated samples from individual stalactites are indicated by commas and replicate numbers. Centroids of the sample sites and variability explained by sampling site are shown. The other stalactite properties (encrustation, water flow) are not visible since they were analyzed as covariates. **a** DCA plot of all the samples. Stalactites selected for 16S rDNA sequencing are shown in *bold*. **b** CCA of all detected TRFs. **c** CCA of TRFs representing at least 10 % of total peak area in at least one sample. **d** CCA of TRFs related to organisms representing ≥20 % of sequences in at least one sample. Sample site abbreviations: ZH-A (samples A), ZH-B (samples B), ZH7, ZH10—Zlaté Hory; MI—Mikulov; BSa, b—Banská Štiavnica, acidic spring; BNa, b—Banská Štiavnica, neutral spring; KU—Kristýnov; indexed sample names (i.e., A12-1) label duplicated samples from a single stalactite

Table 2 Quantity of OTUs representing ≥20 % of sequences in at least one sample and overview of physical properties of the stalactites. Samples marked A and B belong to sample groups ZH-A and B, respectively, samples BS and BN were collected at Banská Štiavnica, MI at Mikulov, and KU in Kristýnov mine. Physical characters and approximate dripping rates of the stalactites are shown. The numbers in the rows of bacterial OTUs represent their percentual proportions in the samples

Sample	BNb	BNa	BSa	KU2	MI1	ZH10	ZH7	A01	A12-1	A12-2	A14-1	A14-2	A03	A07	A04	A05	B04	B16	B08	B07	B10	B11	B12	B03
Dripping rate	2	2	2	0	2	3	3	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
Physical character	G	H	GH	H	G	H	G	H	G	G	E	E	G	G	E	G	H	H	H	H	H	H	H	H
Ferrovum myxofaciens OTU001	0	0	0	0.3	95.0	97.3	40.3	16.7	0	0	22.0	15.0	72.7	86.0	1.3	2.0	14.3	13.0	1.0	22.0	0.7	2.0	0.3	1.3
Acidithiobacillus ferrivorans OTU002	0	0	0	0	0	0	40.0	9.3	0	0	2.3	0	0	0	0	0.3	53.7	45.7	34.7	21.3	12.3	14.0	16.7	0
Acidiferrobacter sp. OTU015	0	0	0	0	0	0	0	29.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sideroxydans litotrophicus OTU003	1.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.0	0	0	4.0	69.0	70.0	65.3	33.7
Gallionella capsiferriformans OTU012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.0	0	0	37.0	3.0	0.3	3.0	2.0
Elusimicrobia OTU25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.0	1.3	0	5.7	3.0	5.0	5.3	29.0
Burkholderia sp. OTU007	1.0	20.3	19.0	19.3	0	0	0	0	3.0	1.3	9.7	22.7	0.3	0	1.3	18.0	1.7	4.0	4.7	0	0	0	0	2.3
Bradyrhizobium elkanii OTU011	0.3	5.0	13.7	23.3	0	0	0	0	1.3	0.3	1.7	2.0	0	0	1.0	7.3	0	1.3	0.7	0.7	0	0	0	1.3
Sediminibacterium sp. OTU17	0.3	24.3	11.3	12.0	0	0	0	0	1.0	3.3	2.0	4.7	0.3	0	0.3	1.3	1.7	3.3	1.3	1.0	0	0	0	4.7
Acidiphilium sp. OTU008	0	0	0	0	0	0	0	0	44.0	35.3	0	0	0	0	3.0	3.7	1.0	3.0	19.0	0	0	0	0	1.3
Acidocella sp. OTU010	0	0	0	0	0	0	0	2.3	17.3	32.0	0.7	0	1.0	0	5.0	19.0	5.0	0	6.7	0	0	0	0	4.0
Metallibacterium sp. OTU022	0	0	0	0	0	0	0.7	26.0	0	0.3	10.0	1.0	4.3	4.0	12.3	0	0	0	1.7	0	0	0	0	0
Metallibacterium sp. OTU101	0	0	0	0	0	0	0.7	5.3	4.0	4.0	9.0	0.7	2.3	0	41.7	4.0	0	0	0	0	0	0	0	0
Thiobacillus aqaesulis OTU060	42.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Others	54.7	50.3	56.0	45.0	5.0	2.7	18.3	11.0	29.3	23.3	42.7	54.0	19.0	10.0	34.0	44.3	9.7	28.3	30.3	8.3	12.0	8.7	9.3	20.3

Legend:

Dripping rate: 0—hanging drop, no dripping observed; 1—rare dripping; 2—intensive dripping; 3—continuous flow

Physical character: H—hard, mineral; G—gelatinous; E—gelatinous, secondarily encrusted; GH—gelatinous growths on a mineral core

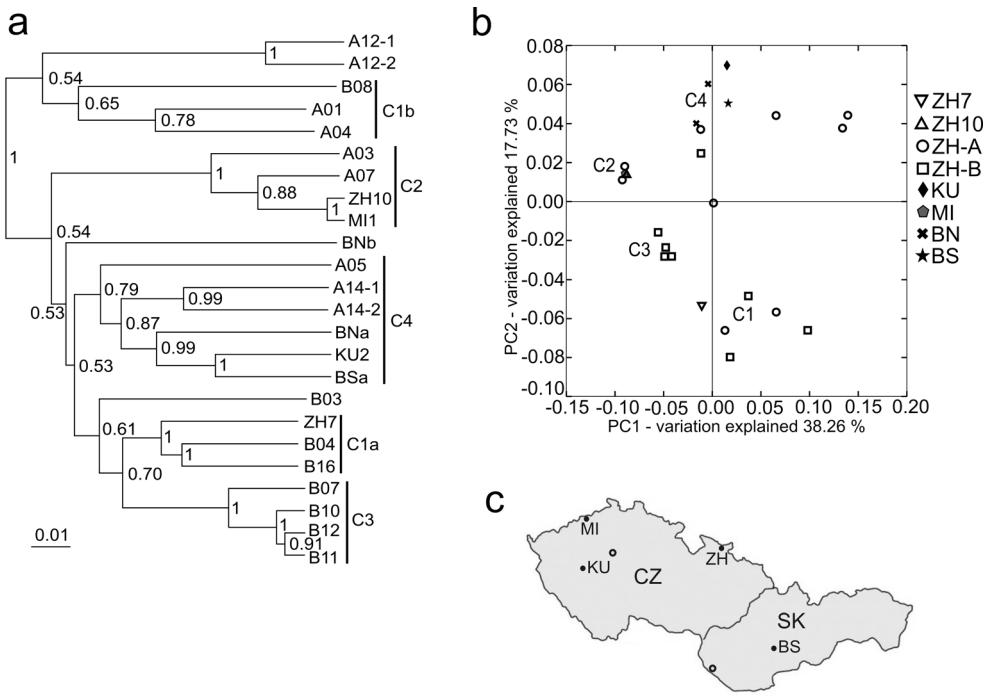

Fig. 2 Results of 16S rDNA amplicon sequencing of selected samples. **a** UPGMA clustering of the microbial communities based on weighted normalized UniFrac metrics. Jackknife values higher than 50 % are shown at the nodes (B). **b** PCoA analysis of the microbial communities based on weighted normalized UniFrac metrics. **c** Map showing locations of the sampling sites. Legend: Sample site abbreviations: BS—Banská Štiavnica; CZ—Czech Republic; KU—Kristýnov mine; MI—Lehnschafter mine, Mikulov; SK—Slovakia; ZH—Zlaté Hory. Clusters of similar communities revealed by the UniFrac analyses are labeled C1 (with sub-clusters a and b), C2, C3, and C4

were found only at sites with elevated water flow. The other clusters, C1, C3, and C4, contained microbial communities from both, high- and low-flow sites.

When unweighted UniFrac, which neglects the abundances of OTUs, was used for clustering the samples, only stalactites from C3 sharing also the sampling site remained partially clustered. The other samples clustered roughly by sites, but generally with low statistical support (Fig. S2B).

Discussion

Variability of the Habitats

The aim of this study was to uncover if processes independent on environmental filtering may deeply influence microbial community structure. These processes are commonly elusive due to two phenomena: (i) practical impossibility to detect all relevant environmental variables and subsequently clearly distinguish environmental filtering and other sources of dissimilarity [35]; and (ii) mixing of numerous physically separated microhabitats within a single sample, which commonly leads to examination of the average microbial community instead of multiple real ones [1]. We tested the possibility to circumvent these issues by sampling strategy. The main stalactite groups ZH-A and B were sampled from sites where water from a single source drips at multiple spots, and thus, more stalactites developed in close vicinity there. Therefore, each stalactite hosted a microbial community supplied by the same water but physically separated from the others. Thus, each stalactite had an individual history. Unfortunately, the existence of minor but important differences between the stalactites (e.g., due to changes in water flow) may not ever be excluded even in a single group. This impossibility was circumvented by adding samples from other undoubtedly more varied sites. If communities from distant sites repeatedly revealed greater similarity than those from adjacent stalactites, the explanation solely by environmental traits would be highly improbable. For this reason, we added stalactites from both geographically and environmentally distant AMD outlets to the sample set. Samples ZH7 and 10 were collected at sites near to ZH-A and B, where, however, the water flow was higher by an order of magnitude [20]. Sample MI was influenced by naturally present As, samples from Banská Štiavnica grew at elevated temperature, and the most divergent site BN was an outlet of ferrous and sulfate-rich but non-acidic water.

The internal homogeneity of each stalactite is as important as the similarity of the stalactites from a single site. The whole stalactite is formed by the same process, and longitudinal water flow maintains only a moderate gradient of the main dissolved compounds [19]. The chemoautotrophic microbial communities inhabiting such environments are known to be conspicuously homogenous in space and time, as was shown by Kay et al. [7] for the assemblage of *F. myxofaciens* and *A. ferrivorans*, which frequently flourish in well-aerated ferruginous acidic mine water outlets and streams with pH between 2 and 3. Similarly, the iron-oxidizing microbial consortia from mine water treatment plants are stable despite variable composition of the water input [36]. Macalady et al. [9] revealed considerable stability of the autotrophic,

sulfur-oxidizing bacterial biofilms growing in sulfidic water outlets in the Frassasi cave system. These biofilms may be formed by several distinct types of microbial communities, which do not mix, even though the conditions below the surface of a biofilm of a certain type can be more suitable for another type. To examine the possibility that the stalactites host homogeneous communities, we sequenced two independent samples from stalactites ZH-A12, A14, and BN. In stalactites ZH-A 12 and 14, the replicated samples were more similar to each other than to samples from different stalactites. The only exception was the stalactite BN, which consisted of two both visually and biologically distinct parts (Table 2, Fig. 2).

Microbial Communities in the Stalactites

Even though certainly not all environmental variables were examined, our experimental design circumvented this constraint by comparing adjacent and thus similar samples with those from distant and dissimilar sites. Both phylogenetic and abundance-based clustering of the microbial communities obviously depended on the presence or absence of a small number of highly abundant OTUs. Some of these OTUs were closely related to typical chemoautotrophs, and they probably represented primary producers in the stalactite ecosystem (*F. myxofaciens*, *A. ferrivorans*, *Sideroxydans litotrophicus*, *Gallionella capsiferriformans*, *Acidiferrobacter* sp.). On the other hand, some stalactites were dominated by presumably heterotrophic bacteria, similarly as in the previous study from Zlaté Hory deposit [20]. Considering the currently available data, it is impossible to decide whether the autotrophs disappeared due to insufficient water flow (excluded for samples BN and BS) or unknown spatial/temporal dynamics of the ecosystem. Nevertheless, it should be noted that a heterotrophs-only community cannot independently persist or even synthesize the organic mass of a snottite in the organic-poor AMD.

Importantly, the majority of the highly abundant species were detected at multiple sites, and, concurrently, various individual stalactites belonging to both groups ZH-A and B were dominated by various species of bacteria. The TRFLP data covering all 51 stalactites were used to test a possible association between the organisms and localities. The variability explained by sampling site was high up to 30.8 %, but the inferred optima for most TRFs and OTUs were not associated with specific sites (Fig. 1b–d). The TRFs, except for those of *T. aquaesulis* and several unidentified minor peaks, were neither sampling site-specific nor followed any monitored environmental variable.

Because a large unexplained variability of the communities was detected, it is important to exclude that it was generated by PCR or sequencing bias, insufficient depth of sequencing, or other artifacts. It is widely accepted that amplicon sequencing yields not only presence/absence data of particular OTUs but also their approximate quantities [37]. Nevertheless, we verified the reproducibility of the methods used in the study. The PCR-tRFLP analyses of most samples were repeated twice, always with high similarity of the replicates (data not shown). Also, the sequencing of replicated samples from certain stalactites showed good reproducibility of the method (see above; Table 2, Fig. 2 a, b). The depth of sequencing was probably insufficient to reveal the complete diversity of rare species in some samples (Table S2). However, most statistical methods used in the community comparison can correctly detect dissimilarities even at a sampling depth of 100 sequences per sample [37]. We analyzed samples with the depth greater than 300 sequences per sample, and the observed patterns were based on substantial variation in abundance of common OTUs and even the absence of certain OTUs in a particular stalactite and their dominance in others (Tables 2 and S2). Such patterns are unlikely to be generated by sequencing bias or undersampling of the rare species.

The amplicon sequencing of the samples from Zlaté Hory mine showed that the microbial community in each acidic ferruginous stalactite may display almost no similarity to the microbial communities in the adjacent stalactites, and that every single stalactite contains only a subset of organisms that can thrive in such environment and are present in the mine. Some stalactites from sites ZH-A and B were even more similar to stalactites from other mines than to the adjacent ones. It is in contradiction to the widely accepted conception, stating that microbial community composition is dictated by environment or by combination of environment and dispersal limitations. It can be objected that the rate of dripping varied considerably even within the stalactite groups ZH-A and B. The dripping rate was not assessed with accuracy sufficient to test its statistic correlation with microbial community composition. However, the same dominant species were found among the samples that differed in water flow by an order of magnitude in both groups ZH-A and B. Clustering analysis by UniFrac confirmed the similarity of these communities (Fig. 2, e.g., ZH7, B04, and B16; B07 and B10; BSa and KU2; ZH10 and MI1). When the stalactites are arranged by dripping rate semi-quantitatively into the four categories (Table 2), different microbial communities appear in each category, and similar communities appear in the different categories as well. The dripping rate (i.e., the number of drops per minute) was considered to be sufficiently accurate estimate of the water flow, because all stalactites had similar area of the terminus and thus produced drops of similar volumes. The differences in dripping rate between stalactites hosting similar communities were securely greater than the possible uncertainty of any estimation method. For these reasons, we conclude that the dripping rate neither determines nor restricts the microbial community composition. More interestingly, all the

clusters of similar communities, apart from C3, contained samples from multiple sites within Zlaté Hory mine or even samples from the other mines as well. It implies that the clustering of the microbial communities does not follow geographic distance, temperature, pH, and concentration of toxic elements (As, Cu). The only exception was the sample BN, which contained a neutrophilic microbial community, almost completely different from the other samples (e.g., *T. aquaesulis*, Fig. 1d). The number of samples was not sufficient to test whether the abovementioned environmental variables influence the probability of the presence of certain organism at a certain site. Nevertheless, the distribution of the community types among the sites (Fig. 2a, b) suggests two different scenarios. Either, some of the unidentified environmental factors, likely the water composition, have a greater spatial variability within the continuous water film originating from a single rock crevice at sites ZH-A and B than among various springs. Or alternatively, the composition of the communities is only delimited, but not determined, by the environmental traits, and thus may considerably vary even at almost identical habitats. From the hydrogeological point of view, the latter explanation is significantly more plausible.

Ecological Implications

These results urge the question whether the influence of the environmental traits can be retrieved from the composition of the stalactite microbial communities. We pooled the communities from randomly selected triads of stalactites from sites ZH-A or ZH-B and analyzed them by UniFrac. Interestingly, the communities from the triads of stalactites from a single site clustered together (Fig. 3), while the pooling never overlapped. It indicates that the pooled microbial communities show stronger relation to the environment than the individual ones. The simplest explanation is that the pooling suppresses the influence of random processes. This explanation should be confirmed by further research performed on substantially greater set of samples. It supports the theory that environmental traits influence probability whether various individual species will or will not be included in the community, rather than set the frequencies of the species in community containing all suitable organisms.

Our interpretation is not in conflict with findings that microorganisms have unimodal response to the environment and that biological responses to environmental gradients can be retrieved by analyzing the phylogenetic dissimilarity of microbial communities [38]. A recent study showed that bacteria evince more deterministic community composition than larger and more dispersal-limited fungi at the newly emerging habitats [2]. It also concurs with our findings, since these results imply that stochastic processes play a significant role in both bacterial and fungal community assembly. On the other hand, numerous studies focusing on AMD-related environments surprisingly overlooked the effects of neutral processes. Likely, it may be explained by sampling of bulky environments that were non-homogeneous at micro-scale, such as sediments and massive biofilms on submerged surfaces [16, 39–41], or by sampling of diverse rather than similar habitats within a single site [17, 20]. Neither of these sampling strategies enables comparison of individual microbial communities from similar habitats. It has

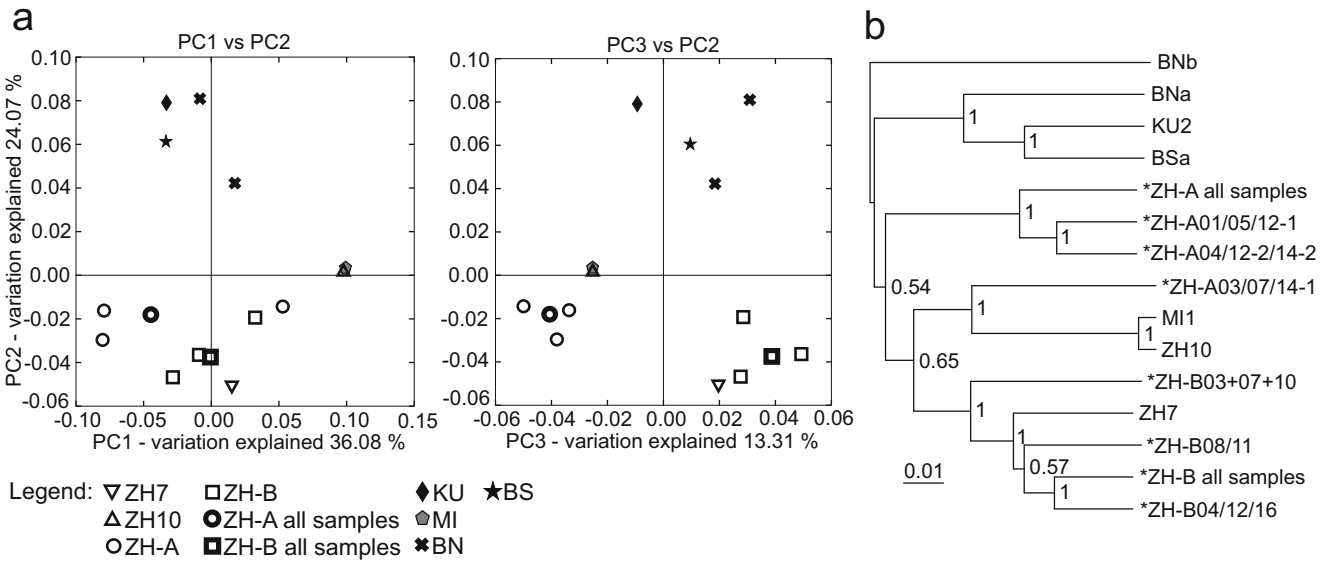

Fig. 3 UniFrac PCoA (**a**) and UPGMA (**b**) clustering of composed microbial communities generated by pooling of three (exceptionally two) randomly selected samples from ZH-A or ZH-B, respectively. Repeated use of any sample in more than one composed community was avoided. Sample site abbreviations are identical to those in Fig. 1. Pooled samples in (**b**) are marked with an *asterisk* (*), and the names of original samples are listed. All composed communities were subsampled to 1000 sequences prior to analysis in order to avoid potential bias caused by unequal sample sizes

been recently shown that the dissimilarity of AMD-related extremophilic microbial communities might be underestimated also due to low sequencing depth in older studies. Even the seemingly identical biofilm communities dominated by *Leptospirillum* sp. revealed distinct populations of rare species when the whole-community transcriptomes were analyzed [42]. It shows that more microorganisms than was previously expected can thrive in such conditions, and that only part of them is present at a specific site at a specific time.

Conclusions

We observed unexpected dissimilarity of microbial communities thriving in ferruginous mine stalactites. Due to the spatial arrangement of the samples, this dissimilarity can be hardly explained by environmental variability. The important question for further research is whether the observed dissimilarity is restricted to habitats such as the microbial stalactites or represents a generally widespread phenomenon. If the present results will be corroborated in multiple various ecosystems, the widely accepted assumption that microbial species alternate in samples primarily due to environmental gradients should be applied with greater caution for extremophilic communities. The subsequent question is whether the common, highly diversified microbial communities (e.g., revealed by most soil metagenome studies) show robust response to the environmental gradients due to mixing of multiple microcommunities in each sample. The mixing supposedly reduces the noise generated by stochastic community assembly. The first step to answer this question may be to test if such typical communities will be obtained by pooling of large numbers of stalactite samples from a single site.

Acknowledgments This work was supported by the Charles University in Prague (project GA UK 528613) and Charles University specific research grant no. SVV 260 208/2015.

We thank to Jan Černý for providing molecular biology laboratory, Petr Baldrian for providing the 454 sequencing facility and help with sample preparation, Jan Kotris for enabling prospection of the Zlaté Hory mine, Pavel Chaloupka for enabling prospection of the Lehnschafter mine, Tomáš Herben and Jana Škodová for the significant help with statistical methods, Petr Drahota for chemical analyses, and Johana Rotterová for thorough language correction.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Nemergut DR, Schmidt SK, Fukami T, O'Neill SP, Bilinski TM, Stanish LF et al (2013) Patterns and processes of microbial community assembly. Microbiol Mol Biol Rev 77:342–356
2. Schmidt SK, Nemergut DR, Darcy JL, Lynch R (2014) Do bacterial and fungal communities assemble differently during primary succession? Mol Ecol 23:254–258
3. Nemergut DR, Lozupone C, Jiang L, Schmidt SK, Fierer N, Townsend AR et al (2011) Global patterns in the biogeography of bacterial taxa. Environ Microbiol 13:135–144
4. Hallberg KB, Coupland K, Kimura S, Johnson DB (2006) Macroscopic streamer srowths in acidic, metal-rich mine waters in North Wales consist of novel and remarkably simple bacterial communities. Appl Environ Microbiol 72:2022–2030
5. Macalady JL, Jones DS, Lyon EH (2007) Extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy. Environ Microbiol 9:1402–1414
6. García-Moyano A, Gonzáles-Toril E, Aguilera Á, Amils R (2012) Comparative microbial ecology study of the sediments and the water column of the Río Tinto, an extreme acidic environment. FEMS Microbiol Ecol 81:303–314
7. Kay CM, Rowe OF, Rochetti L, Coupland K, Hallberg KB, Johnson DB (2013) Evolution of microbial "streamer" growths in an acidic, metal-contaminated stream draining an abandoned underground copper mine. Life 3:189–210
8. Baker BJ, Banfield JF (2003) Microbial communities in acid mine drainage. FEMS Microbiol Ecol 44:139–152
9. Macalady JL, Dattagupta S, Schaperdoth I, Jones DS, Druschel GK, Eastman D (2008) Niche differentiation among sulfur-oxidizing bacterial populations in cave waters. ISME J 2:590–601
10. Johnson DB (2012) Geomicrobiology of extremely acidic subsurface environments. FEMS Microbiol Ecol 81:2–12
11. Golyshina OV, Pivovarova TA, Karavaiko GI, Kondratéva TF, Moore ER, Abraham WR et al (2000) *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the *Ferroplasmaceae* fam. nov., comprising a distinct lineage of the *Archaea*. Int J Syst Evol Microbiol 50:997–1006
12. Hallberg KB, Gonzáles-Toril E, Johnson DB (2010) *Acidithiobacillus ferrivorans*, sp. nov.; facultatively anaerobic, psychrotolerant iron-, and sulfur-oxidizing acidophiles isolated from metal mine-impacted environments. Extremophiles 14:9–19
13. Schippers A, Breuker A, Blazejak A, Bosecker K, Kock D, Wright TL (2010) The biogeochemistry and microbiology of sulfidic mine waste and bioleaching dumps and heaps, and novel Fe(II)-oxidizing bacteria. Hydrometallurgy 104:342–350
14. Hallberg KB, Hedrich S, Johnson DB (2011) *Acidiferrobacter thiooxydans*, gen. nov. sp. nov.; an acidophilic, thermo-tolerant, facultatively anaerobic iron- and sulfur-oxidizer of the family Ectothiorhodospiraceae. Extremophiles 15:271–279
15. Johnson DB, Hallberg KB, Hedrich S (2014) Uncovering a microbial enigma: isolation and characterization of the streamer-generating, iron-oxidizing, acidophilic bacterium "*Ferrovum myxofaciens*". Appl Environ Microbiol 80:672–680
16. Amaral-Zettler LA, Zettler ER, Theroux SM, Palacios C, Aguilera A, Amils R (2011) Microbial community structure across the tree of life in the extreme Rio Tinto. ISME J 5:42–50
17. Kimura S, Bryan CG, Hallberg KB, Johnson DB (2011) Biodiversity and geochemistry of an extremely acidic, low-temperature subterranean environment sustained by chemolithotrophy. Environ Microbiol 13:2092–2104
18. Casamayor EO, Pedrós-Alió C, Muyzer G, Amann R (2002) Microheterogeneity in 16S ribosomal DNA-defined bacterial populations from a stratified planktonic environment is related to temporal changes and to ecological adaptations. Appl Environ Microbiol 68:1706–1714
19. Ziegler S, Ackermann S, Majzlan J, Gescher J (2009) Matrix composition and community structure analysis of a novel bacterial pyrite leaching community. Environ Microbiol 11:2329–2338

20. Falteisek L, Čepička I (2012) Microbiology of diverse acidic and non-acidic microhabitats within a sulfidic ore mine. Extremophiles 16:911–922
21. Patočka F, Vrba J (1989) The comparison of strata-bound massive sulfide deposits using the fuzzy linguistic diagnosis of the Zlaté Hory deposits, Czechoslovakia, as an example. Miner Dep 24: 192–198
22. Martyčák K, Zeman J, Vacek-Veselý M (1993) Supergene processes on ore deposits—a source of heavy metals. Environ Geol 23: 156–165
23. Turner S, Pryer KM, Miao VPW, Palmer JD (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J Euk Microbiol 46:327–338
24. Větrovský T, Baldrian P (2013) Analysis of soil fungal communities by amplicon pyrosequencing: current approaches to data analysis and the introduction of the pipeline SEED. Biol Fertil Soils 49: 1027–1037
25. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB et al (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541
26. Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461
27. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
28. Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066
29. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704
30. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M et al (2008) The Metagenomics RAST server—a public resource for the automatic phylogenetic and func-tional analysis of metagenomes. BMC Bioinformatics 9:386
31. ter Braak CJF, Šmilauer P (2002) CANOCO Reference manual and users guide to Canoco for Windows: software for canonical community ordination (Version 4.5). Microcomputer Power, Ithaca
32. Hill T, Lewicki P (2007) Statistics methods and applications. StatSoft, Tulsa, URL http://www.statsoft.com/textbook/stathome.html
33. Hamady M, Lozupone C, Knight R (2010) Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. ISME J 4:17–27
34. Chao A, Chazdon RL, Colwell RK, Shen T-J (2005) A new statistical approach for assessing similarity of species composition with incidence and abundance data. Ecol Lett 8:148–159
35. Ramette A, Tiedje JM (2007) Multiscale responses of microbial life to spatial distance and environmental heterogeneity in a patchy ecosystem. PNAS 104:2761–2766
36. Heinzel E, Janneck E, Glombitza F, Schlömann M, Seifert J (2009) Population dynamics of iron-oxidizing communities in pilot plants for the treatment of acid mine waters. Environ Sci Technol 43: 6138–6144
37. Kuczynski J, Liu Z, Lozupone C, McDonald D, Fierer N, Knight R (2010) Microbial community resemblance methods differ in their ability to detect biologically relevant patterns. Nat Methods 7:813–819
38. Faith DP, Lozupone CA, Nipperess D, Knight R (2009) The cladistic basis for the phylogenetic diversity (PD) measure links evolutionary features to environmental gradients and supports broad applications of microbial ecology's "phylogenetic beta diversity" framework. Int J Mol Sci 10:4723–4741
39. Bond PL, Smriga SP, Banfield JF (2000) Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. Appl Environ Microbiol 66:3842–3849
40. Rowe OF, Sánchez-España J, Hallberg KB, Johnson DB (2007) Microbial communities and geochemical dynamics in an extremely acidic, metal-rich stream at an abandoned sulfide mine (Huelva, Spain) underpinned by two functional primary production systems. Environ Microbiol 9:1761–1771
41. Tan GL, Shu WS, Zhou WH, Li XL, Lan CY, Huang LN (2009) Seasonal and spatial variations in microbial community structure and diversity in the acid stream draining across an ongoing surface mining site. FEMS Microbiol Ecol 70:121–129
42. Aliaga-Goltsman DS, Comolli LR, Thomas BC, Banfield JF (2015) Community transcriptomics reveals unexpected high microbial diversity in acidophilic biofilm communities. ISME J 9:1014–1023