Extremophiles (2012) 16:911–922
DOI 10.1007/s00792-012-0488-2

ORIGINAL PAPER

Microbiology of diverse acidic and non-acidic microhabitats within a sulfidic ore mine

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Received: 6 April 2012 / Accepted: 27 September 2012 / Published online: 12 October 2012

Abstract A wide variety of microhabitats within the extremely acidic abandoned underground copper mine Zlaté Hory (Czech Republic) was investigated. SSU rDNA libraries were analyzed from 15 samples representing gossan, sulfide-leaching environments in the oxidation zone, and acidic water springs in the mine galleries. Microbial analyses were extended by analyses of chemical composition of water and solid phases and identification of arising secondary minerals. The microbial communities of the three main classes of microenvironments differed in almost every aspect. Among others, ecological partitioning of *Acidithiobacillus ferrooxidans* and the recently described *A. ferrivorans* was observed. Distinct types of communities inhabiting the water springs were detected. The more extreme springs (pH <3, conductivity >2 mS/cm) were inhabited by "*Ferrovum*" spp. and *A. ferrivorans*, whereas *Gallionella* sp. dominated the less extreme ones. A new role for gossan in the extremely acidic ecosystem is proposed. This zone was inhabited by a large diversity of neutrophilic heterotrophs that appeared to be continuously washed out to the acidic environments localized downstream. Five species originating in gossan were found in several acidic habitats. Here they can survive and probably serve as scavengers of dead biomass, particularly from chemoautotrophic growths. No such process has been described from acidic mine environments so far.

Keywords Acidophiles · Chemolithotrophy · Microbial consortia · Phylogeny · Gossan · Supergenic processes

Communicated by M. da Costa.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-012-0488-2) contains supplementary material, which is available to authorized users.

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Introduction

Ecosystems based on oxidation of sulfides have been attracting the attention of scientists for decades. Moreover, they have large economical potential (Pecina et al. 2010; Puhakka et al. 2007). Since sulfides, and especially pyrite, are very common minerals in the Earth's crust, numerous extremely acidic environments associated with sulfidic mineral deposits and sulfur-rich water springs can be found. Many studies have focused on describing biodiversity of acidic drainages from sulfidic mines and on the characterization of the most important bacterial species (e.g. Golyshina et al. 2000; Johnson et al. 2001; Okabayashi et al. 2005; Hallberg et al. 2006; Tan et al. 2007; Heinzel et al. 2009; Xiao et al. 2009; Hallberg et al. 2010, 2011). The influence of the season, the depth of the sample in the sediment and the distance of the sampling site from the mine adit on microbial community composition have been thoroughly investigated (Rowe et al. 2007; Tan et al. 2009; Kimura et al. 2011). These efforts resulted in a discovery of a large number of bacterial species involved in the main biogeochemical processes in extremely acidic environments. The association of various processes with particular sets of bacterial species is thus well established and the diversity of acidophiles, although generally rather low, has been described in more detail than the microbial diversity of many less extreme environments. However, most of the studies have been limited to a single or a few

sampling sites similar to each other within the locality. Yet numerous distinct habitats can be found in a single sulfidic ore mine. The habitats differ primarily in the presence of rock dissolution, in water composition, the presence of bacterial streamers, sediment formation, etc. (Kimura et al. 2011). Another factor which may increase the complexity of the mine ecosystem, but which is usually neglected in geomicrobiological studies, is the presence of the leached zone (gossan). This zone forms the uppermost part of the deposits (Enders et al. 2006). Virtually all water infiltrating the deposit has to pass through gossan. During this passage water may become enriched in certain chemical compounds (Romero et al. 2011). The degree to which gossan affects water composition depends on its thickness and on the way the water passes through (e.g., single, highly permissive fracture vs. filtration through altered rock) both of which influence the retention time and the intensity of contact with gossan. The role of microorganisms in mobilization of silver from gossan has been reported recently (Izawa et al. 2010).

It could be expected that bacterial communities inhabiting various microenvironments within the sulfidic ore deposit vary considerably. It has been well established that the true extent of natural habitats of certain microbes cannot be inferred solely from their requirements in laboratory culture. Although the total diversity of prokaryotes at an ore deposit is dictated mainly by its acidic character, the diversity at a particular microenvironment is driven mostly by the biological processes, like competition or mutualism (Johnson 1998). Ecosystems of sulfidic ore deposits contain numerous types of habitats whose diversity is influenced not only by interactions within a single habitat, but also by interactions with other habitats. The factors shaping microbial diversity should be studied in detail since the composition of microbial communities influences the geochemical processes significantly (e.g. Johnson 1998). However, only few authors have focused on bacterial consortia at various types of habitats within a single locality, or in various microenvironments within a single sampling site (Schrenk et al. 1998; Espana et al. 2007; Macalady et al. 2007; Amaral-Zettler et al. 2011; Kimura et al. 2011).

The combination of bacteriological, mineralogical and chemical analyses can provide deep insight into the processes taking place at particular sites. Certain bacterial species may even serve as indicators of particular biochemical processes (Baker and Banfield 2003; Mendez et al. 2008). Despite major progress in the knowledge of the ecology of various bacterial species as well as whole bacterial communities, the understanding of the sulfidic ecosystem in its complexity remains a challenge.

In the present study, we have chosen a sampling scheme that differed from most of the published works. The large abandoned sulfidic mine Zlaté Hory, with highly acidic character, has been explored in order to assess the variability of its habitats. Non-acidic sites in the subsurface part of the deposit were also included. Composition of the microbial communities is discussed in the context of local geochemical conditions.

Materials and methods

Site description

The Zlaté Hory deposit is located in Silesia (Czech Republic) in a mountain region (50°12–13'N, 17°23–24'30″E) and reaches an elevation of 540–974 m a.s.l. It is composed of sulfidic ores dispersed in metamorphic Devonian volcano-sedimentary rocks, mostly quartzites, quartzitic shales and metatuffites. The impregnation type of mineralization dominates throughout the deposit (Vaněček 2004). The deposit is strongly altered by oxidative weathering of sulfides. Typical leached zone (gossan) with heavy metals (Fe, Cu, Zn, Pb) present in the form of oxides, sulfates and other oxidized secondary minerals can be found in depths of up to hundred meters below the surface. This zone contains low-mineralized and pH-neutral water. Various secondary minerals (goethite, gypsum, alumogel, allophane, linarite, brochantite, malachite, clay minerals) have been observed in the mine works in gossan. Spontaneous leaching of sulfidic ores causing alteration of the ambient rock and massive formation of acidic mine waters (typical pH 2.5–3.5; total mineralization 2–5 g/L) takes place in the oxidation zone. Both neutral and acidic, metal-rich waters rise from various rock fractures, but the proportion of acidic waters is much larger and increases with the depth. Precipitation of various pH-neutral secondary minerals from the less mineralized neutral water occurs in the oxidation zone as well.

The mining district is divided into several deposits. Our study focused on two deposits, ZH-south and ZH-east (Fig. S1). The deposit ZH-south contains monometallic pyrite–chalcopyrite–pyrrhotite Cu ores. The deposit was partially mined out in 1965–1990. The leached and oxidation zones of ZH-east were extracted in several periods from the fourteenth to the nineteenth century. The oxidation zone containing polymetalic Zn–Pb–Ag sulfidic ores with a significant content of pyrite was partially mined out by sublevel chambering during the twentieth century; the deposit ZH-east was abandoned in 1993 (Kotris 2004).

The vertical range of presently accessible mine works reaches 350 m and represents all zones of the deposit from strongly weathered subsurface rock to unaltered ore bodies. The mine is fully spontaneously ventilated and has a stable air temperature of 8–10 °C and water temperature of 8–9 °C in the deep parts throughout the year. In the area close to the surface the water and air temperature may decrease to

4–5 °C in winter period, certain parts of the zero and first haulage levels may experience sub-zero temperatures.

Sample collection

Initially, 28 samples were collected from sites representing a broad spectrum of combinations of pH, conductivity, oxygenation and secondary minerals. After preliminary chemical and biological analyses (SEM-EDS, XRD, sequencing up to 10 SSU rDNA clones), a set of 15 samples representing main types of environments was selected for further examination.

Samples of secondary minerals, rock biofilms and streamers for microbiological analyses were taken into sterile 15-mL tubes (one biological sample per site). Each mineral was also sampled for XRD and EDS analyses. When samples immersed in water were collected, the pH, conductivity, temperature and dissolved oxygen level of the water were measured using WTW Multi303i universal pocket meter (WTW, Germany). The water samples for microbiological and chemical analyses were collected into sterile 1.5-L plastic bottles without disturbing a potential solid phase. The water flow was estimated from the filling times of the sample bottles (the flow was directed into the bottle quantitatively). All samples were delivered to the laboratory within 24 h. For the TEM analysis, small pieces of material (ca. 2 mm) were collected directly into the fixing solution (see below).

Chemical analyses of water

The chemical analyses of the water samples were conducted in the certified commercial analytical laboratory VZ lab s.r.o. (Prague, Czech Republic). Anions were determined using ion chromatography analyzer ICS 1100 (Dionex, USA), total organic carbon (TOC) using the TOC-VCPN (Shimadzu, Japan), Si and humic substances were determined photometrically following the protocol of Horáková et al. (1986), concentrations of metal cations by AAS using SpectrAA 220FS (Varian, USA), chemical oxygen demand by permanganate (COD_{Mn}) by titration, COD_{Cr} by photometry, and total minerals by gravimetry.

Mineralogical analyses

A representative portion of each solid sample was homogenized and analyzed by XRD using X′Pert Pro (PANalytical BV, Netherlands) in Cu Kα at 40 kV, 30 mA, step scanning at 0.05°/250 s in the range 3°–70° 2θ. Mineral phases were identified using the analytical software X′Pert HighScore 1.0d (PANalytical B.V., Netherlands).

Microstructure of carbon-covered solid samples was examined by scanning electron microscopy (SEM) using CamScan S4 (Siemens, Germany) in both the backscattered and secondary electron signal at 15 kV. The elemental composition of various morphologically distinguishable materials was identified using energy dispersive spectroscopy (EDS) microanalytic system Link ISIS 300 (Oxford instruments, GB).

Cryo-FESEM

Samples were transferred into a special holder with a slot and clamping facility, mounted into a drop of glue and clamped. The samples were extremely quickly frozen (>10^3 K/s) in slushy nitrogen. Then, the samples were transferred into the cryo-stage of the preparation chamber (ALTO2500, Gatan, USA) where they were freeze-fractured at −140 °C, freeze-etched at −95 °C for 3 min, and then coated with 3 nm layer of platinum at −135 °C. The coated samples were inserted into the chamber of the JSM-7401F microscope (JEOL, Japan) precooled to −130 °C. Images were obtained by both the secondary and backscattered electron signal at 3 kV.

TEM

Immediately after collection, the sample was transferred into a fixative containing 2.5% glutaraldehyde in 0.1 M SCB (pH 7.2) at mine temperature (ca. 9 °C) and delivered to the TEM laboratory. There, it was postfixed in 2 % OsO_4 in 0.1 M SCB (pH 7.2) at room temperature for 3 h followed by dehydration through ethanol series and substitution with acetone. The sample was embedded in a resin (Epon 812, Polysciences, USA). Ultrathin sections were cut on Leica EM UC6 ultramicrotome (Leica Microsystems) and double-stained with 2% (w/v) uranyl acetate and lead citrate (Reynolds 1963). The sections were observed using JEOL JEM-1011 TEM (Jeol, Japan) with CCD camera Veleta and acquisition software Olympus Soft Imaging Solution (Olympus, Japan).

DNA extraction, amplification, cloning and sequencing

The solid and gelatinous samples were divided by flame-sterilized lancet and forceps. Approximately 250 mg of each sample were used for DNA extraction. 0.5 L of each water sample was filtered using sterile 0.22 μm pore syringe filter (Millipore, USA) and the membrane from the filter was used for DNA extraction. Genomic DNA was isolated using ZR soil microbe DNA kit (Zymo research, USA). The primers U515F (GTGCCAGCMGCCGCGGTAA) and U1406R (GACGGGCGGTGTGTRCA) (Turner et al. 1999) were used to amplify approximately 860-bp-long fragment of the SSU rRNA gene from *Bacteria*, *Archaea*, and mitochondria. The annealing temperature of 55 °C and LA DNA polymerase (Top-bio, Czech

Republic) were used. PCR products were purified using Zymoclean gel DNA recovery kit (Zymo Research, USA), TA cloned into pGEM-T easy vector (Promega, USA) using chemocompetent *Escherichia coli* TOP10 cells. 45–50 clones of each sample were sequenced on 3100 Genetic Analyzer (Applied Biosystems, USA). Newly determined sequences have been deposited in GenBank under accession numbers JQ217496–JQ218118.

Phylogenetic analyses

At first, a preemptive data set containing all 614 newly determined sequences was created. The sequences were aligned using MAFFT (Katoh et al. 2002) with the help of the EBI server (http://www.ebi.ac.uk/Tools/msa/mafft/); both the number of iterations and the number of tree rebuildings were set to 100. Genetic distances (p-distances) of the aligned sequences were computed in PAUP* 4.0b10 (Swofford 2002). Sequences with p-distances lower than 2 % were considered to belong to conspecific organisms. The species identity of up to seven representatives of each putative species was estimated by BLAST. In some cases two already described species with p-distances lower than 2 % were detected (e.g., *At. ferrooxidans* and *At. ferrivorans*). The coverage of microbial diversity was estimated using Chao1-bc diversity estimator (Chao et al. 2005). Four final data sets were created. The first data set consisted of 196 SSU rDNA sequences from GenBank representing broad bacterial diversity and 75 newly determined sequences of non-proteobacterial eubacteria. The second data set consisted of 220 SSU rDNA sequences representing the diversity of *Proteobacteria* and 163 newly determined sequences belonging to *Proteobacteria*; 15 non-proteobacterial sequences were used as outgroups. The third dataset consisted of 40 sequences representing the diversity of main archaeal groups and 11 archaeal sequences from the present study. The fourth dataset consisted of 41 mitochondrial SSU rDNA sequences representing main eukaryotic groups and 7 newly determined sequences; 5 proteobacterial sequences represented outgroup. For divergent sequences (no identified sequence with homology higher than ca. 95 % was found), closest neighbors were found by BLAST and were included in the datasets as well. Sequences from each data set were aligned by MAFFT as described above and the alignments were then manually edited. Phylogenetic trees were constructed by maximum likelihood in RAxML 7.2.6. (Stamatakis 2006) under the GTRGAMMAI substitution model, and were bootstrapped with 1000 replicates.

Statistical methods

Multivariate analyses were run in Canoco 4.5 (ter Braak and Šmilauer 2002). Since the gradient lengths in the data were long, we used unimodal methods. The variability within the dataset was visualized using a correspondence analysis (CA) and a canonical correspondence analysis (CCA) was applied to test whether the differences in species composition are significantly related to the habitat type. We created dummy variables for each of the habitat types. In case of mixed habitat character, a value was assigned to the two relevant dummy variables.

Results and discussion

Surprisingly high diversity of ecological niches and microbial communities has been found within the abandoned sulfidic ore deposit Zlaté Hory. Samples representing three basic types of habitats—(1) gossan or the border zone between gossan and the acidic environment; (2) the oxidation zone where the rock dissolution dominated and (3) the mine gallery springs, where secondary minerals precipitated from extremely acidic waters—were collected (see Fig. S1 for the spatial arrangement of the sampling sites within the mine and Fig. S2 for the typical in situ appearance of bacterial growths and associated secondary mineral accumulations). The essential characteristics of the samples are summarized in Table 1. Six species of *Archaea*, 137 species of *Bacteria* and 5 species of protists were detected; their identification was confirmed by phylogenetic analyses (Figs. S3–S6). More than 50 % of the total diversity estimated by Chao1-bc was identified in all samples except ZH7a and 16, although the confidence intervals were large in more samples.

The proposed clustering of the samples by the three basic types of environments (i.e. gossan, leaching and acidic springs) was confirmed by a correspondence analysis (Fig. 2). An additional type of samples was established during the analyses. Samples of this type were interpreted as acidic spring growths that were undergoing decay at the time of collection (see below). Differences in bacterial communities living in these types of habitats were significant ($p < 0.002$) and the variable habitat explained 33 % of the observed variability.

Non-acidophilic bacterial communities

Virtually all water infiltrates the mine through gossan. As a result, chemical compounds and bacteria are continuously being transported from gossan to the acidic ecosystem of the mine. We have analyzed samples representing water percolating through gossan (ZH16a—a water stream flowing from old stopes through a contemporary chimney), three different sediments found in proximity of this water stream (ZH16, 16b, and 19), and the sediment from a gossan-like islet found deep in the acidic zone (ZH2, Table 1).

Table 1 The basic characterization of sampled materials and mineral phases identified by XRD

Sample	Physical character	Depth below surface (m)	pH	Conductivity (mS/cm)	Dissolved O_2 (mg/l)	Temperature (°C)	Estimated water flow (l/s)	Mineralogy
Gossan								
ZH2	Wall crust	140	NA	NA	NA	9	0	Serpierite-deviline, (hendricksite, gypsum)
ZH16	Wall crust	65	6.45	0.76	10.3	8.4	0.2	99 % amorphous
ZH16a	Water	65	6.45	0.76	10.3	8.4	0.2	–
ZH16b	Wall crust	65	6	NA	NA	8.4	>0.05	Goethite
ZH19	Wall crust	65	6.45	0.76	10.3	8.4	0.05	Schulenbergite, cuprian, hydrowoodwardite, zincowoodwardite, ca. 50 % amorphous
Rock dissolving environments								
ZH6	Corroded rock	125	NA	NA	NA	9	0	Quartz, pyrite, K-feldspar, aluminocopiapite, sulfur
ZH7a	Water	190	2.9	3.7	3.09	8.7	0.1	–
Mine gallery springs								
ZH4	Gelatinous	140	NA	NA	NA	9	0	–
ZH7	Gelatinous stalactite	190	2.9	3.7	3.09	8.7	0.1	Schwertmannite
ZH8	Soda straw stalactite	220	NA	NA	NA	9	Very low[a]	Schwertmannite, (gypsum), 90 % amorphous
ZH9	Pendulous stalactite	220	3	NA	NA	9	Very low[a]	Schwertmannite, goethite
ZH10	Hard stalactite	220	3.08	2.3	7.9	9	0.5	Schwertmannite, (gypsum, goethite), 90 % amorphous
ZH12	Soda straw stalactite	180	NA	NA	NA	9.5	Very low[a]	Schwertmannite, goethite, (gypsum), 90 % amorphous
ZH13	Pendulous stalactite	180	3	NA	NA	9.5	Very low[a]	Schwertmannite, (mica, baryte)
ZH14	Streamer	70	3.1	1.94	7.55	4.5	0.4	Schwertmannite, quartz, (muscovite, gypsum), 90 % amorphous

Minerals with minor proportion are in parentheses

[a] The stalactites were moist from acidic water, but not enough to measure exact values of dissolved oxygen concentration and conductivity

Samples ZH16, ZH19, and ZH2 were various Cu- and Zn-containing secondary minerals formed at circumneutral pH. ZH16 precipitated from the water stream ZH16a. It was the only sample to contain fibers, probably organic, encrusted by alumosilicates (Fig. 1h). ZH19 was a dark blue-green material precipitated from the same stream as ZH16, but at the peripheral zone, which had been washed by water only in high-flow periods. Both ZH16 and 19 contained grains enriched in noble earth elements (Table S2).

Although obviously only a fraction of the true bacterial diversity has been identified in these samples, the overall character of bacterial communities was similar (Fig. 3; Table S1). Aerobic or microaerophilic heterotrophs and facultative or obligatory iron- and nitrite-oxidizing autotrophs dominated gossan. ZH16a was ecologically strikingly uniform. Only aerobic (one aerotolerant) neutrophilic heterotrophic bacteria were detected. Known hydrocarbon degraders were found in ZH16a (*Acinetobacter* spp., *Achromobacter xylosoxidans*, *Burkholderia cenocepacia*; Sette et al. 2007; Boontawan and Boontawan 2011), probably due to an industrial contamination of the mining area. In ZH16, methanogenic *Archaea* indicated the occurrence of anaerobic environments. Frequent iron oxidizers were found in ZH19, which contained almost no iron. Most likely, these bacterial cells were transported to the sampling site from corroding iron supports in the upper parts of the chimney.

ZH16b, which was found at the opposite side of the same chimney as ZH16, differed both chemically (almost pure goethite, Table 1; Table S2) and microbiologically from the other gossan samples. In addition to typical gossan bacteria, a large proportion of acidophilic Fe-oxidizers were found, even though the pH of the sample was neutral (interestingly, the same acidophilic, iron-oxidizing

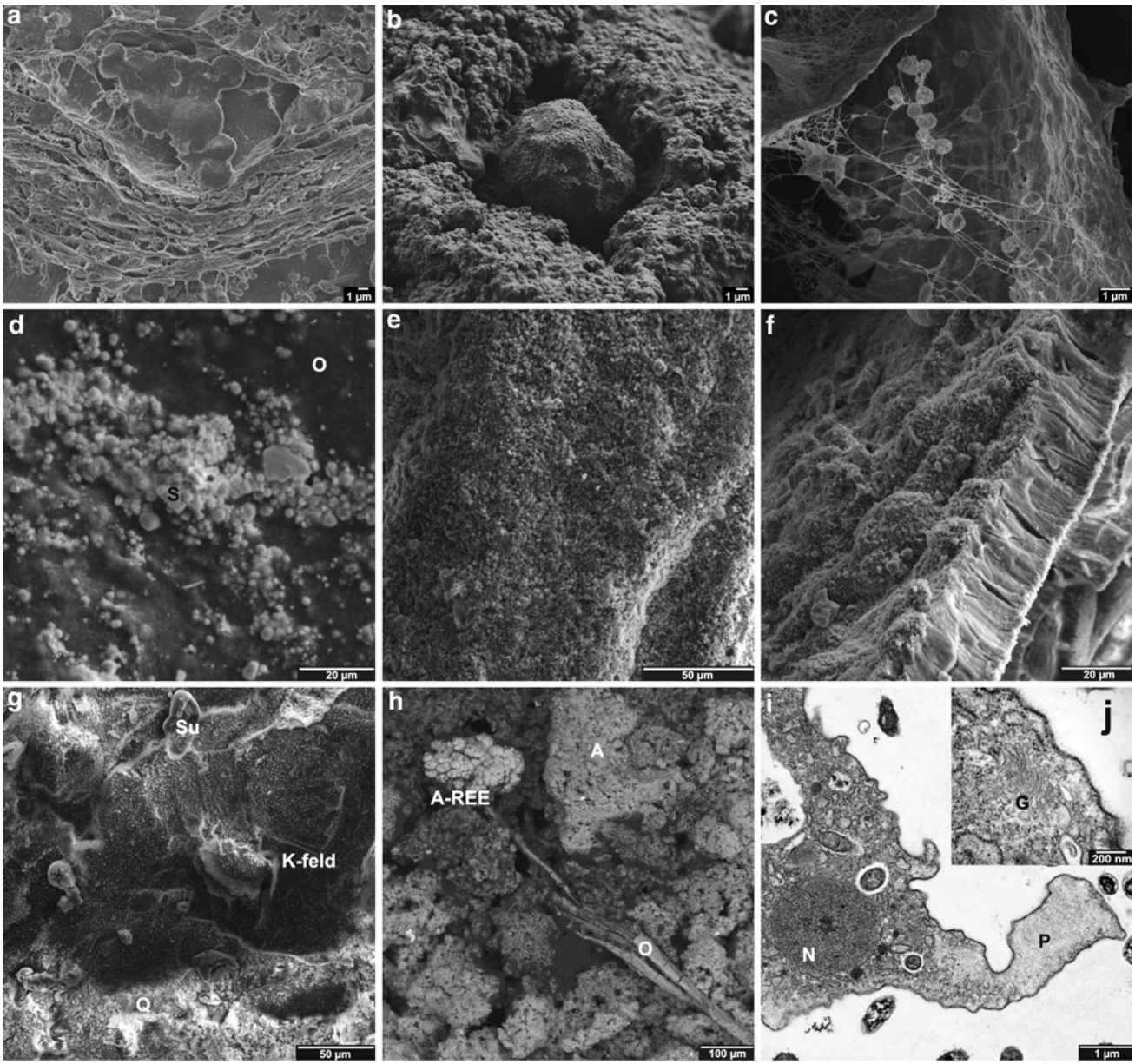

Fig. 1 A typical microstructure of samples as observed by cryogenic (**a**–**c**) and classic (**d**–**h**) SEM and by TEM (**i**, **j**). Minerals were identified by elementary composition and XRD patterns of the samples. **a** Newly formed schwertmannite between bacterial fibers in ZH7. **b** A rock element in the stalactite ZH9. **c** Bacterial cells in the filamentous matrix of ZH13. **d** Mineral domains in the presumably organic material from the gelatinous stalactite ZH7. **e** Schwertmannite–goethite particles covered by presumably organic material in the pendulous stalactite ZH9. **f** A compact schwertmannite crust of the soda straw stalactite ZH12. **g** A corroded surface of the rock ZH6 covered with bacterial cells and sulfur particles. **h** Amorphous Al-, Si- and Cu-rich material of the sample ZH16 with an encrusted, probably organic, fiber and Mn- and Ce-enriched grain. **i**, **j** Transmission electron microscopy of an unidentified lobose amoeba found in the sample ZH7 *A* amorphous Si- and Al-rich material, *A-REE* amorphous material enriched in noble earths, *K-feld* altered K-feldspar, *O* organic material, *Q* quartz, *S* schwertmannite, *Su* sulfur, *arrows* glycocalyx, *G* Golgi body, *N* nucleus with central nucleolus, *P* pseudopodium with ectoplasm

archaeal species were found in ZH7a—water from a site where pyrite dissolution occurred; Fig. 3). Our interpretation is that ZH16b differed from the other gossan samples in the source of water. The water flowing through samples ZH16 and 19 originated in gossan associated with old mine works above the chimney. In contrast, the water flowing through ZH16b originated in a modern mining chamber containing an altered ore pile, a possible source of acidic mineralized water. This water would then get neutralized on its way to the chimney. Unfortunately, the water path could not be explored since the outset of the chimney is inaccessible today.

Rock dissolution sites

The habitats of this type were represented by samples ZH6 and ZH7a (Table 1). The sample ZH6 represented strongly corroded quartz rock that had been located in a talus pile in a large mine chamber for 30–40 years. The SEM-EDS examination of a rock cavity from ZH6 showed frequent bacterial cells on the quartz, pyrite, and altered K-feldspar surface. Newly formed grains of elemental sulfur of up to 25 μm in diameter were observed (Fig. 1g). The cavities were partially filled with secondary minerals. Although the volume of the sample ZH6 was not sufficient to perform XRD, an analysis of a very similar material collected from another piece of rock in the same waste pile indicated gypsum and aluminocopiapite. None of the prokaryotes from ZH6 (*At. ferrooxidans*, *F. acidiphilum*, *L. ferrooxidans*) were found in any other sample. These prokaryotes were shown to tolerate lower pH values than bacteria found in less extreme habitats (Bond et al. 2000; Golyshina and Timmis 2005; Hallberg et al. 2010). In ZH6, *At. ferrooxidans* replaced the recently described *At. ferrivorans*, which was found at the other habitats. A possible explanation is the higher extremity of the rock surface. Although the pH value of ZH6 could not be determined due to an insufficient amount of water, we suppose it was extremely low, probably the lowest from all the samples. For decades, the air moisture and possibly minute amounts of splashing water in extremely wet periods had been the only source of water, so the water film covering the sample was extremely concentrated. Remarkable corrosion of K-feldspar and even quartz together with a formation of an acidic evaporite aluminocopiapite (Buckby et al. 2003) support this hypothesis.

ZH7a was collected from a water spring running through a rock crack in the same mine chamber where ZH6 was found (Fig. S1). It represented one of the most acidic water springs in the mine, with high content of sulfates, Fe, Al and Cu (see Table 2). Interestingly, $COD_{Mn,Cr}$ was determined several times in 2009 and 2010 and was continuously high with a maximum of 257 mg/l during dry periods.

In contrast to ZH6, the sample ZH7a was dominated by an uncultured *Thermoplasmatales* archeon, which was recently found in various acidic iron-rich waters (e.g. Amaral-Zettler et al. 2011; Kimura et al. 2011), and by Fe- and S-oxidizing *Proteobacteria* which indicated the sulfide dissolution as the main process in the rock fracture. Acidophilic heterotrophic *Dokdonella* sp., possibly acidotolerant *Stenotrophomonas maltophilia* and five species related to poorly described environmental samples were also detected. Elevated concentrations of Al and Si in ZH7a indicated that acidic leaching of alumosilicate minerals occurred in the rock surrounding the fracture (see Dopson et al. 2007).

Table 2 The composition of water from the samples ZH7/7a, 14 and 16/16a

Sample	ZH7a	ZH14	ZH16a
pH	2.7	2.9	7.3
Conductivity (lab, mS/cm)	3.78	1.48	0.649
Ammonia	0	0	0
Nitrites	0	0	0
Nitrates	2.7	0	2.7
Chlorides	8.3	3.6	130
Sulfates	3509	797	68.5
Fluorides	5.1	0.93	0.13
Na	2.9	1.8	55.1
K	2.5	0.91	0.91
Ca	49.7	40.5	66.1
Mg	97.4	21.8	14.9
Fe	774	57.3	0
Mn	26.7	4.4	0.07
Si	18	17	4.7
COD_{Mn}	71.2	1.6	2.6
Total mineralization	5805	915	–
Phosphates	0	0	0
Humic substances	0	0	0
TOC	7.7	4.9	–
As	0.225	0.044	0
Ba	0.82	0.63	2.4
Be	0.0039	0.001	0
Al	131	19.9	0
Cr	0.11	0	0
Cd	0.048	0.007	0
Co	1.5	0.19	0
Cu	90	7.6	0.08
Ni	0.74	0.12	0
Pb	0	0	0
Ag	0	0	0
Zn	5.9	0.91	0.37
Li	0.09	0.023	0

All concentrations, TOC, COD and total mineralization are in mg/l

Bacterial communities of the two rock dissolution environments were unique among the examined samples, although the same main biogeochemical reaction (oxidation of Fe^{2+}, Rawlings 2002) was found at many other sites. The only similar community was found in ZH16b, where rock dissolution was also proposed (Figs. 2, 3). This is the first report of ecological differences in situ between *Acidithiobacillus ferrooxidans* (ZH6) and *A. ferrivorans* (ZH7 and 8, Fig. 3). Our data support the laboratory culture results of Hallberg et al. (2010).

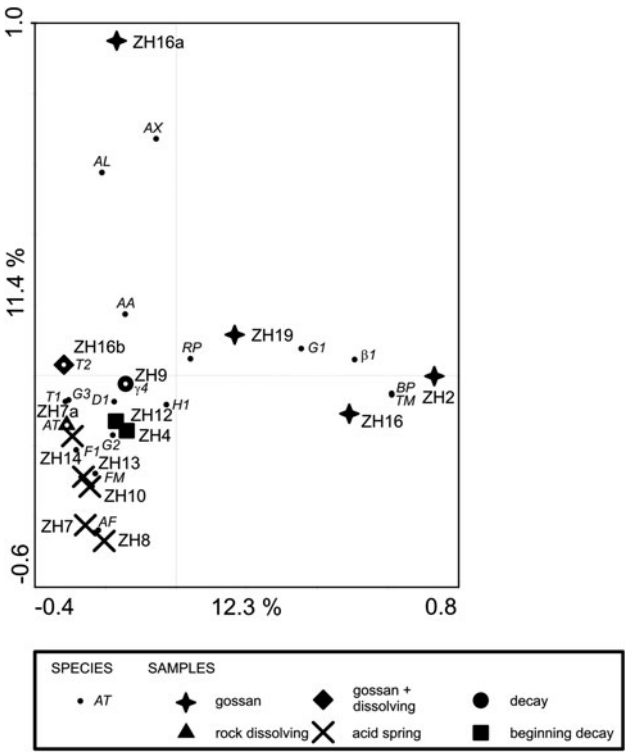

Fig. 2 Correspondence analysis showing clustering of samples into groups corresponding to the main types of environments. Sample ZH6 was left out since it shared no species with other samples and disrupted the clustering of other samples. The dissolving environment ZH7a seems to cluster with acidic springs, but it clearly separated from them and clustered with ZH16b in the third dimension (data not shown). Optimal positions of selected important species are shown. *β1 Betaproteobacteria* OTU1, *γ4 Gammaproteobacteria* OTU4, *AA Acidovorax avenae*, *AF Acidiferrobacter thiooxidans*, *AL Acinetobacter lwofii*, *AT Acidithiobacillus ferrooxidans*, *AX Achromobacter xyloxidans*, *BP Burkholderia phenazinium*, *D1 Dokdonella* sp. OTU1, *F1* "*Ferrovum*" sp. OTU1, *FM* "*Ferrovum myxofaciens*", *G1 Gallionella* sp. OTU1, *G2 Gallionella* sp. OTU2, *G3 Gallionella* sp. OTU3, *H1 Herbaspirillum* sp. OTU1, *RP Ralstonia picketii*, *T1 Thermoplasmatales* sp. OTU1, *T2 Thermoplasmatales* sp. OTU2, *TM Thiohalomonas* sp

Extremely acidic waters in mine galleries

A dramatic change occurs when water gets from the rock cracks to the mine gallery. It loses its contact with the rock serving as a source of iron and sulfuric acid, but also as a source of neutralizing agents. Accumulation of schwertmannite, goethite and gypsum is common in mine gallery environment. The acidic water springs are represented by samples ZH4, 7, 8, 9, 10, 12, 13, and 14 (Table 1). Our results show a surprising diversity of habitats and bacterial communities in water springs located within a relatively small territory (Fig. 3 Fig. S1).

As observed during the preliminary site prospection, massive gelatinous growths were abundant in springs with pH lower than 3 and conductivity greater than 2 mS/cm. They were missing in places where pH increased or conductivity decreased as a result of mixing with contaminating water. In springs with significant water flow (samples ZH7, 10, 14, and a decaying stalactite ZH4) bulky hard or gelatinous stalactites often develop, while at places where acidic water drops slowly (samples ZH8, 13, and decaying stalactites ZH12 and 9), hard soda straw-like stalactites and thin pendulous stalactites are usually formed (Fig. S2, compare with Kimura et al. 2011).

ZH7 was a gelatinous stalactite growing in the water spring sampled as ZH7a. ZH7 consisted mostly of rod-shaped bacteria, usually arranged in fibers, accompanied by mineral grains with predominating schwertmannite (Fig. 1a, d). Microbial community was dominated by iron-oxidizing autotrophs, "*Ferrovum myxofaciens*" and *At. ferrivorans*, and resembled acid streamers described in Hallberg et al. (2006). The same bacterial species also predominated in the streamers growing in highly mineralized acidic water streams in proximity of ZH7 (data not shown). A single clone of a neutrophile was also detected in ZH7 (Fig. 3). Examination by TEM revealed two morphotypes of rod-shaped bacteria and an amoeba with numerous vacuoles containing symbiotic or phagocytized bacteria (Fig. 1i).

Another high-flow spring stalactite, ZH10, grew in highly mineralized water, with pH slightly higher than 3, which was flowing out of a crack in the ceiling. The microbial community of ZH10 differed substantially from ZH7, with predominating iron-oxidizing autotrophic bacteria *Gallionella* sp., "*F. myxofaciens*", and an uncultured *Acidimicrobiaceae* bacterium (Fig. 3; Table S1). Similarly to the other stalactites where the water pH was higher than 3, ZH10 was formed by porous granular schwertmannite, which was probably layered. The massive gelatinous growths were not observed (Fig. S2a–d).

Another set of conditions, i.e. water with pH fluctuating around 3, but with significantly lower concentrations of the main ions, was found in ZH14. This sample was a gelatinous streamer found right next to a spring which was impossible to sample directly for technical reasons. The water composition was similar to ZH7a, but approximately six times diluted. COD_{Mn} was 44,5× lower in ZH14 (Table 2). A greater diversity of iron-oxidizing autotrophs, including a novel "*Ferrovum*" species, diverse acidophilic, mostly aerobic or facultatively anaerobic, heterotrophs and a single non-acidophile (*Acinetobacter lwofii*) was detected (Fig. 3; Table S1). The increasing diversity of bacteria is consistent with the decreasing extremity of the habitat (Bond et al. 2000).

These findings underline the essential role of pH and mineralization in the composition and morphology of bacterial communities, even when the energy source, the terminal metabolic products, the hydrodynamic

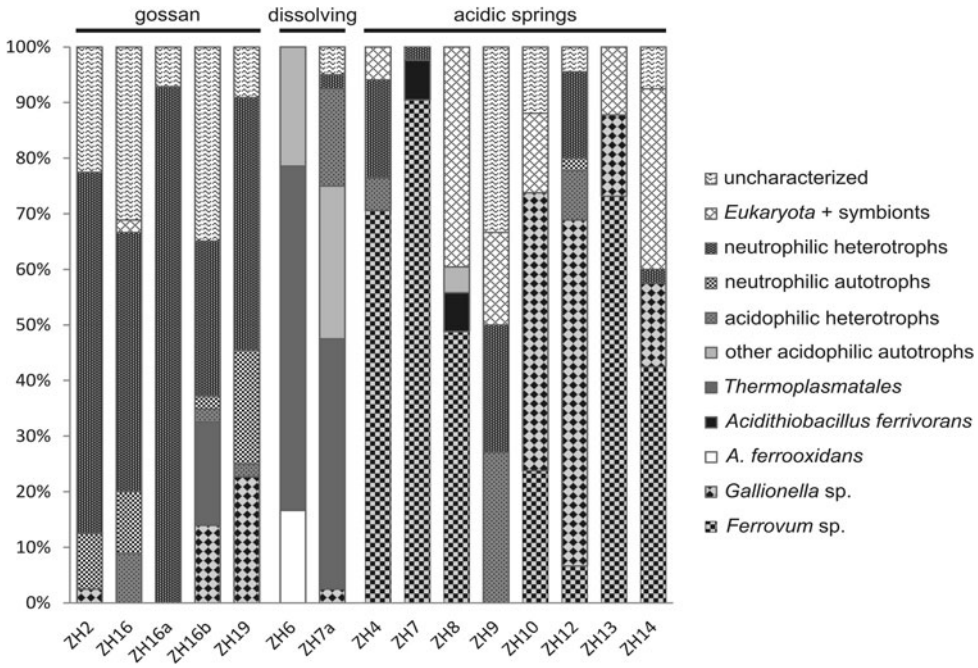

Fig. 3 Comparison of main microbial ecological groups and selected genera in the Zlaté Hory microhabitats. For characteristics of the samples see Table 1

parameters, and the main biogenic or toxic elements are almost identical. The ecological niches of the bacterial consortia were much more limited than would be assumed from the known growth requirements of particular bacterial species in the laboratory culture. The most likely explanation is that competition limits the occurrence of every species to habitats with optimal physico-chemical conditions (Johnson 1998; Bond et al. 2000).

Eukaryotic organisms and bacteria considered to be their symbionts were detected in all acidic spring samples (Fig. 1i, j). The eukaryotes probably belonged to the Amoebozoa, although the statistical support was sufficient for some clones only (Fig. S6). Eukaryotes are probably common constituents of acidic metal-rich environments (Amaral-Zettler et al. 2002; Macalady et al. 2007).

In order to complete the spectrum of analyzed water streams, we examined four stalactites from places where the water drops slowly from the minor rock cracks (ZH8, 9, 12, 13). Two of the stalactites, one soda straw-like (ZH8) and one pendulous (ZH13), showed simple communities similar to the growths in high-flow highly acidic and mineralized waters (e.g. ZH7, Fig. 3). However, the number of cells per typical visual field of cryo-FESEM was approximately ten times lower (Fig. 1a, c). Additionally, one clone of a divergent amoebozoan and a divergent amoebal endosymbiotic bacteria belonging to *Parachlamydiaceae* and *Rickettsiales* were detected.

Another soda straw stalactite (ZH12) hosted a community of *Gallionella* sp., "*F. myxofaciens*" and four other autotrophs as the primary producers, together with five heterotrophs. Some of them were not typical acidophiles (Table S1). Despite macroscopic similarity to ZH8, the microstructure of ZH12 was different. The SEM observation showed quiescent crystallization of layered schwertmannite indicating a slower rate of encrustation in ZH12 (Fig. 1f), whereas ZH8 had more chaotic porous structure. We conclude that the low-flow stalactites host microbial associations dependent on the chemoautotrophic oxidation of iron by the primary producers. These associations clustered with the high-flow communities in CA analysis (Fig. 2) while no significant difference was detected by CCA.

The microbiota of the pendulous stalactite ZH9 was completely different. Typical acidophilic autotrophs were replaced by multiple species of acidophilic and neutrophilic heterotrophs. The clones were affiliated to various taxonomic groups including candidate groups TM6 and WS6. *R. picketii*, *Acidovorax avenae* and *Herbaspirillum* sp. represented the most abundant neutrophilic species (Fig. 3; Table S1). The microstructure of ZH9 differed from the other gelatinous samples. In contrast to samples ZH7 and 13, where the organic matrix was fibrous and porous, with numerous freshly formed schwertmannite beads and bacterial cells (Fig. 1a, c), the structure of ZH9 was compact, without any visible cells or the bulky organic matrix (Fig. 1b, e). It was composed of schwertmannite–goethite in Fe:S ratio varying from 9.1 to 11.7. The proportion of goethite was thus markedly higher than in all other high-flow (Fe:S 2.8–3.9) and low-flow stalactites (Fe:S 4.4–7.7). Therefore, the mineral material of ZH9 must have been older than that of other samples since schwertmannite passes to goethite spontaneously as a result of aging (Schwertmann and Carlson 2005). The most likely explanation of the unique character of ZH9 is that the organic matrix enclosing mineral grains was decomposing at the time of sampling.

These observations are consistent with the growth phase of stalactites being followed by the decay when the supply of ferrous iron becomes insufficient. The primary producers then disappear and are replaced by heterotrophic bacteria decomposing the organic matrix of the stalactite. Alternate explanation that the stalactites does not decay but simply grow at less extreme sites is not applicable for many reasons. Mainly because the less extreme but not decaying samples ZH10 and 14 were inhabited almost strictly by acidophiles that were mostly autotrophic (Fig. 3).

This hypothesis was also supported by the analysis of ZH4. This sample was a fragment of gelatinous stalactite similar to ZH7 found at the bottom of a mining chamber. Most likely, it had fallen down from the mining chamber shortly before the collection and thus is in the beginning phase of decay. If our hypothesis is correct, a mixture of bacteria resembling ZH7 and 9 should be detected in ZH4. Its microbial community consisted of "*F. myxofaciens*" accompanied by *R. picketii* and the same species of *Acidobacteria* and amoebae as in ZH9 (Table S1).

Stalactites in several different phases of the growth–decay cycle were sampled in the recent study: samples ZH7, 8, 10, and 13 represented growing stalactites, ZH4 and 12 were in the initial phase of decay, and ZH9 was in the advanced decay phase after colmatage of its tributary rock cracks. Our observations are most similar to the results of Kimura et al (2011) showing heterotrophic bacteria flourishing at the bottom of gelatinous acidic water streamers. There, the low supply of ferrous iron and oxygen limited the autotrophs and dissimilative iron reduction may have been favored. However, the microbial community differed from the communities in our present study and no decaying stalactites were explored.

Interestingly, a large portion of the bacteria contributing to the decay in Zlaté Hory mine were not typical acidophiles. Furthermore, five out of ten species of non-acidophilic heterotrophs found at the acidic habitats were abundant in gossan, including the most frequent *R. picketii*, *A. avenae*, and *Herbaspirillum* sp. (Fig. S4; Table S1). Moreover, we expect that the observed proportion of heterotrophs common for both gossan and the acidic zone would increase with more thorough gossan sampling. Such a phenomenon has not yet been reported from acidic environments, although acidotolerant isolates of *Sphingomonas* sp. and *R. picketii* were described recently (Kimura et al. 2011). Due to the high content of heavy metals in gossan, the bacteria should be preadapted to the metal-rich environment. Heavy metal-resistant strains of *Ralstonia*, *Herbaspirillum*, *Burkholderia*, and *Acinetobacter* have already been reported (Sun et al. 2010; Yang et al. 2010).

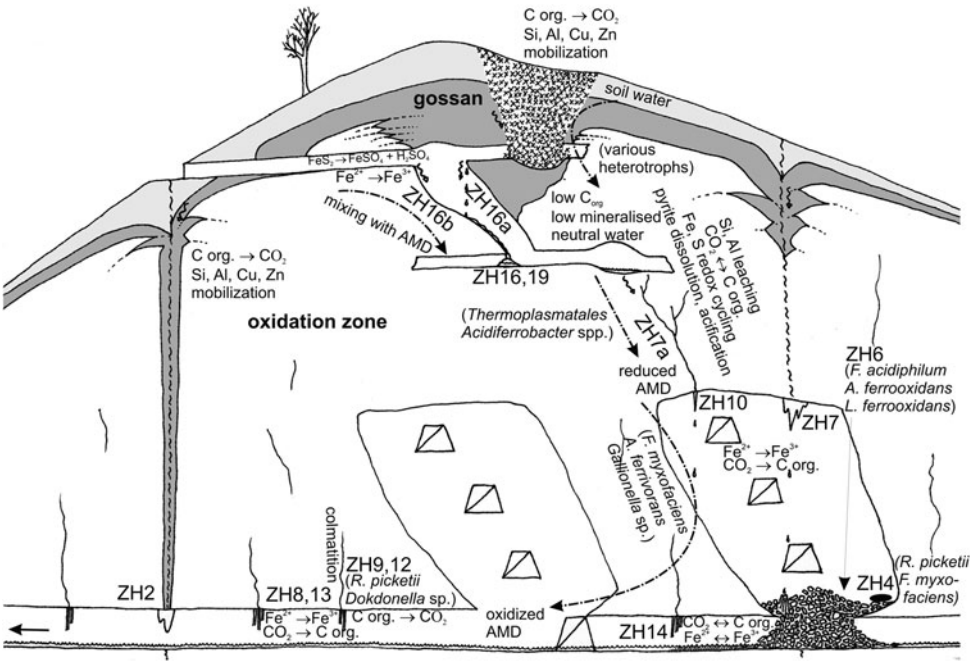

Fig. 4 A scheme showing the main processes, types of habitats and bacterial species identified in the Zlaté Hory mine ecosystem. Mainly aerobic oxidation of organic compounds, mobilization and redeposition of heavy metals, Si and Al occurs in gossan (*dark grey*). The organics-depleted drainage from gossan percolates in the fissures of the oxidation zone (*white*), where oxidative dissolution of sulfides by chemolithotrophic bacteria occurs. Water becomes acidic and enriched in Fe^{2+} (reduced AMD). Autotrophic iron-oxidizing bacterial growths flourish at the places where water discharges from fissures to the mine gallery and in the drainage streams. Heterotrophic bacteria are continuously imported to these growths and may overgrow the autotrophs, especially when the source of ferrous iron is lost due to the local changes in water circulation

We hypothesize that the bacteria living in gossan are continuously being washed out and imported to the deeper zones of the deposit. Here some of them survive the acidic conditions, become part of the ecosystem and serve as decomposers of the organic material. Although non-acidophilic heterotrophs constitute only a minor part of growing acidic streamers, they may overgrow the acidophiles in the decay phase. The adaptation of the bacteria to low pH could be explained by prolonged coexistence of acidic and neutral zones in close proximity. The boundary between the zones represents an optimal environment for adaptation of bacteria. Thus, the heterotrophic bacteria originating in gossan should be recognized as a novel component of the sulfide weathering ecosystem.

Concluding remarks

Geochemical processes maintain distinct microbial communities in the three main zones of the ecosystem (i.e. gossan, the rock dissolution sites in the oxidation zone, and the mine gallery springs; Figs. 2, 4) despite the water flow that continuously transports bacterial cells from gossan to the oxidation zone and from the oxidation zone to the mine gallery springs. In the mine gallery springs, several different consortia of autotrophic bacteria sharply delimited by pH and mineralization grow next to each other (Fig. 4). At the same time, some of the gossan bacteria preadapted to the acidic and toxic conditions and are able to survive even in acidic environment, where they decompose decaying growths of acidophilic bacteria. Stable import of gossan bacteria to the acidic ecosystem may be an important factor influencing the biodiversity and functions of the whole ecosystem. Thus, the role of gossan in the sulfidic ore deposit weathering should not be neglected because it has the potential to change both the chemical and the microbiological processes taking place in the deposit.

Acknowledgments This work was supported by Institutional Research Concept MSM0021620858 and the Czech Science Foundation project (P506/11/1317) and SVV (project SVV-2012-265 206). We thank Jan Černý for providing laboratory facilities; Jan Kotris (Diamo, RD Jeseník) for enabling prospection of the site; Václav Krištůfek for providing access to the Cryo-FESEM facility; Petr Drahota and Radek Procházka for XRD analyses; Linda Nedbalová for statistical analyses; Marie Mikšaníková and Tomáš Machač for graphic design of some illustrations and Naděžda Brdičková for language corrections. The authors also wish to thank anonymous reviewers for providing a thorough and thoughtful critique of the manuscript.

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