Chemolithotrophic microbial mats in an open pond in the continental subsurface – implications for microbial biosignatures

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The 450 m deep Äspö Hard Rock Laboratory (Äspö HRL), run by the Swedish Nuclear Fuel and Waste Management Co. (SKB) offers a unique opportunity to access microbial systems within Precambrian, mostly granodioritic rocks of the Baltic Shield. Biofilms and microbial mats at a deep groundwater seepage site and an associated pond exhibit a large diversity of aerobic and anaerobic, often chemolithotrophic microorganisms. An open pond system consisting of several different subsystems was studied to explore the diversity and spatial distribution of microbial communities and associated mineral precipitates. A further focus was placed on the establishment of inorganic biosignatures (especially trace and rare earth element (TREE) fractionation patterns) for biogeochemical processes involving subsurface microorganisms.

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Introduction

Biofilms cover almost every rock-water interface on Earth. Within biofilms and microbial mats, microorganisms often mediate (bio-)mineral formation due to their presence and metabolism that cause changes in the chemical equilibrium of the surrounding environment. While biologically induced mineralization pathways can be well specified in theory, they are often difficult to recognize and distinguish in natural samples. Nevertheless, such biogenic processes may produce minerals different from their inorganically formed varieties in shape, size, crystallinity, isotopic and trace element composition (Konhauser 1997; Ferris et al. 1999, 2000; Weiner & Dove 2003, Bazylinski et al. 2007; Haferburg & Kothe 2007; Takahashi et al. 2007; Templeton & Knowles 2009; Chan et al. 2011). Such inorganic ‘biosignatures’ may be specified and utilized for the identification of related biological processes in geological samples, even when organic biosignatures, namely lipid biomarkers, have not survived over geological time.
A better understanding of mineralizing microbial communities in the subsurface may furthermore provide insights and analogies to early Earth ecosystems. Early life on Earth probably developed in the subsurface, where it was protected from meteorite impacts and radiation penetrating the newly forming atmosphere (Trevors 2002; Russel 2003; Bailey et al. 2009).

In this study, mineralizing microbial mats were investigated from an open pond located at ca. 160 m depth in the Åspö Hard Rock Laboratory (HRL; north of Os-
Karshamn, Sweden). The Åspö HRL was built as a testing environment for the disposal of nuclear waste and is operated by the Swedish Nuclear Fuel and Waste Management Co (SKB). In the Åspö HRL, a wide spectrum of chiefly chemolithotrophically driven subsurface ecosystems is accessible (Pedersen 1997) that may offer a window into the microbial biosphere existing in continental vein systems. The study pond at the Åspö HRL site NASA 1127B (Fig. 1) exhibits a broad diversity of aerobic and anaerobic microorganisms, with iron/manganese oxidizing consortia forming a several cm thick microbial mat (Fig. 2). In our study, we paid particular attention to ion species involved in microbial mineralisation processes. To distinguish selective cation incorporation, three distinct types of microbial mats and the overlying water body were systematically analysed, and the in- and out-flows of the pond were compared. Especially trace and rare earth element (TREE) fractionation patterns were investigated to assess their potential utility as inorganic biosignatures for individual groups of microorganisms.

Methods

Microbial mats

Microbial mats from the pond (Fig. 1A) were separated in three visually different mat types, (ochre, white and black). Whereas the ochre mat was mainly distributed over the whole pond, exhibiting a cauliflower structure (Figs. 1D, E), the black and the overlying white mat were contiguously distributed along the rim and at some localised spots in the pond (Figs. 1B, C). The mats were sampled with different devices, depending on the mat consistency, using spatulas, petri dishes and syringe cores. Additionally, an ochre mat core was taken with a plexiglas tube of 15 cm Ø and 25 cm length (Fig. 2). After sampling, the microbial mats were frozen, transported with dry ice, and stored at -20°C and -80°C (samples for DNA analysis) until further processing.

Chemical analysis of the pond water and the microbial mats

Oxygen in the feeder fluids was measured using the Winkler method (Hansen 1999). Anion concentrations, measured by titration and ion chromatography, conductivity, pH and spectrophotometrical Fe$_{total}$/Fe(II) data were analysed immediately after sampling by the certified SKB chemistry lab.

For sample conservation and TREE measurements, concentrated, distilled HNO$_3$ was added to 50 ml water samples (final concentration 2 % HNO$_3$). In order to quantify the amounts of REE in the microbial mats, 4 ml of H$_2$O$_2$ and 2 ml of concentrated, distilled HNO$_3$ were added to 500 mg of lyophilised sample. The resulting solutions containing the dissolved mineral precipitates were diluted in 50 ml of deionised water (final concentration 4 % HNO$_3$). These solutions, a reference sample (blank) containing all chemicals used, and the water samples were spiked with internal Ge, Rh, In and Re standards and analysed by ICP-MS and ICP-OES. As a reference, Fe$_{total}$ was also measured by ICP-OES and was in good agreement with the spectrophotometrical data (1 % deviation). TREE were analysed using Inductive Coupled Plasma Mass Spectrometry (ICP-MS; Perkin Elmer SCIEX Elan DRCII) and Optical Emission Spectroscopy (ICP-OES; PerkinElmer Optima 3300 DV).

Carbon (C$_{tot}$), total nitrogen (N$_{tot}$) and sulphur (S$_{tot}$) were analysed using a CNS Elemental Analyser (HEKAtech Euro EA). C$_{inorg}$ and organic carbon (C$_{org}$) were analysed with a Leco RC 412 multiphase carbon analyzer. Double measurements were performed routinely, and appropriate internal standards were used for the C$_{inorg}$ (Leco 502-030), C$_{org}$ (Leco 501-034), and CNS (BBot and atropine sulphate, IVA Analysentechnik SA990752 and SA990753) analyses.

Microsensor measurements

The microenvironment was probed using glass microsensors for O$_2$, pH and H$_2$S as previously described in (Santergoeds et al. 1998; Wieland et al. 2005). Four different environments were chosen for analysis: (1) Fresh looking ochre coloured mats in the upper part of the pond; (2) Aged mats of darker colour in the lower part of the pond; (3) Small black mat spots in the lower part of the pond; (4) Large black mat zone containing a white microbial mat in the corner of the lower part of the pond.

![Fig. 2: Push core of an iron oxidizing microbial mat taken from the central part of the pond. The colour zoning and the different layers within the profile are attributed to different mat generations.](image-url)
Fig. 3: Microsensor profiles into the ochre mat (A) and the black and white mat (B). The oxygen (O$_2$) concentration at different measurement points in the ochre mat decreases much faster in dense mat parts (continuous line) than in more fluffy mat parts (dashed lines, A). The O$_2$ and pH profiles penetrating the black and white mats show at first a roughly stable profile in the water column. At the mat surface (7 cm water depth) a sharp drop in O$_2$ concentration is visible, with a concurrent rise of the H$_2$S concentration and a pH shift by 0.1 (B).

**Confocal Laser Scanning Microscopy (CLSM)**

Structural characterization and localization of microbial cells and associated EPS structures within the mineralized ochre mats were examined by Laser Scanning Microscopy using a TCS SP1 confocal laser scanning microscope (Leica, Heidelberg, Germany).

Biofilm organisms were stained using nucleic acid-specific (Syto 9; Sybr Green) and protein-specific fluorochromes (Sypro Red). In addition, lectin-binding analysis was employed for the characterization of EPS glycoconjugates (PSA_Alexa568).

**Fluorescence in situ hybridization (FISH)**

Loosely attached microbial biofilms were carefully sampled by means of petridiscs with the aim to minimize biofilm destruction. Samples were fixed with 4% paraformaldehyde (final concentration) for 4 hrs and carefully transferred to 1x PBS (10 mM sodium phosphate; 130 mM NaCl) three times before a final storage in 50% ethanol/1x PBS at -20°C until further processing. Hybridization and microscopy visualisation of hybridized and 4',6-diamidino-2-phenylindole (DAPI)-counterstained cells were performed as described previously (Snaidr et al. 1997). Cy3- and Oregon green monolabeled oligonucleotides were purchased from Metabion (Martinsried, Germany). Probes and formamide (FA) concentrations used in this study were as follows: EUB338 I-III targeting bacteria (Daims et al. 1999; 35% FA), Non338 (negative control; Wallner et al. 1993; 10% FA), Beta42 targeting Beta-Proteobacteria (Manz et al. 1992; 35% FA), SRB385 targeting sulfate reducing bacteria (SRB) inclusive most species of the δ-group of purple bacteria (Amann et al., 1990; 35% FA) and DSS658 targeting Desulfosarcina spp./Desulfococcus spp./Desulfofrigus spp. and Desulfofaba spp. (Manz et al. 1998; 35% FA). Background signal of samples, observed with the nonsense probe NON338, was negligible (<0.1%).

**454 analysis**

A DNA sample of the fresh looking ochre mat was sent for Roche 454 pyrosequencing at the MrDNA lab (Shallowater, TX, USA). Sequencing of the 16S rRNA gene was done using the 28F and 519R general primers (Lane 1991). 6500 sequences were obtained and were processed as described in Ionescu et al. (2012). Shortly, the sequences were checked for quality (length, homopolymers and ambiguous bases) and aligned against the SILVA ref database. Sequences with poor alignment score were removed while the rest were dereplicated and clustered (per sample) into operational taxonomic units (OTUs) at a similarity value of 98%. Reference sequences of each OTU were assigned to a taxonomy using BLAST against the SILVA ref database (Version 111; Quast et al. 2013). Full OTU mapping of the sample was done by clustering the reference sequences of each OTU at 99% similarity.

**Scanning Electron Microscopy and Energy Dispersive X-ray analysis (SEM-EDX)**

For SEM-EDX analysis, the fresh samples were immediately fixed in 2% glutaraldehyde and stored at 4°C until analysis. Prior to measurement, the samples were dehydrated in rising ethanol concentrations in 15%, 30%, 50%, 70% ethanol (30 min each), followed by 90% and 99% (60 min each), and 99% ethanol (12 hrs). After the dehydration series, the samples were mounted on SEM sample holders and sputtered with Au-Pd (7.3 nm for 120 sec). Samples were analysed using a field emission SEM (LEO 1530 Gemini) combined with an INCA X-act EDX (Oxford Instruments).
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Fig. 4: SEM micrographs of the orange microbial mat, dominated by iron oxidizing bacteria (IOB). The microbial mat is visibly dominated by stalks produced by *Gallionella ferruginea* and *Mariprofundus* sp. (A). Iron oxyhydroxide precipitates resembling Metallogenium-like structures were occasionally observed (B). *Mariprofundus/Gallionella* EPS stalks show different stages of iron oxyhydroxide impregnation, most likely as a function of cell activity and age (C). The iron oxyhydroxide structures are connected via filigree structures similar to nanowires (D; Reguera et al. 2005).

Fig. 5: SEM micrographs exhibiting different kinds of microbial cells and EPS structures of the white (A, B) and the black (C, D) microbial mats, mainly composed of rod shaped and spirally coiled cells; (A) rods, filaments, (B) rods, spirillum, (C) rods, (D) spirochaetes.
Table 1: Major physicochemical parameters of the pond water (NASA 1127B).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1127B inlet</th>
<th>1127B main</th>
<th>1127B outlet</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO₄ µg/l</td>
<td>44.8</td>
<td>54.8</td>
<td>50.4</td>
</tr>
<tr>
<td>NO₃</td>
<td>1.9</td>
<td>2.7</td>
<td>11.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
<td>7.3</td>
<td>7.2</td>
</tr>
<tr>
<td>kH₄, mS/m</td>
<td>1090</td>
<td>1074</td>
<td>1075</td>
</tr>
<tr>
<td>Alkalinity, mg/l</td>
<td>184.8</td>
<td>173.9</td>
<td>166.6</td>
</tr>
<tr>
<td>Uranine, %</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Cl⁻ mg/l</td>
<td>3461</td>
<td>3405</td>
<td>3409</td>
</tr>
<tr>
<td>Br⁻ mg/l</td>
<td>15.8</td>
<td>15.2</td>
<td>14.5</td>
</tr>
<tr>
<td>SO₄²⁻ , mg/l</td>
<td>333.6</td>
<td>346.3</td>
<td>346.5</td>
</tr>
<tr>
<td>F mg/l</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Ca²⁺ mg/l</td>
<td>147.0</td>
<td>146.7</td>
<td>147.9</td>
</tr>
<tr>
<td>Na⁺ mg/l</td>
<td>1521</td>
<td>1511</td>
<td>1537</td>
</tr>
<tr>
<td>Mg²⁺ mg/l</td>
<td>141.4</td>
<td>143.4</td>
<td>145.1</td>
</tr>
<tr>
<td>K mg/l</td>
<td>36.0</td>
<td>40.3</td>
<td>40.5</td>
</tr>
<tr>
<td>Fe tot mg/l</td>
<td>1.59</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Fe(III) mg/l</td>
<td>1.51</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Mn mg/l</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Al mg/l</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Sr mg/l</td>
<td>6.7</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Ba mg/l</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Si mg/l</td>
<td>4.5</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>O₃ mg/l</td>
<td>0.7</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td>H₂S mg/l</td>
<td>0.04</td>
<td>0.08</td>
<td>0.02</td>
</tr>
</tbody>
</table>

DNA extraction, PCR amplification and Denaturing Gradient Gel Electrophoresis (DGGE)

Genomic DNA was extracted directly from environmental subsamples by using the Ultra Clean Soil DNA extraction kit (MOBIO Laboratories, Inc., CA) according to the manufacturer’s protocol.

Partial 16S rRNA gene amplification from environmental DNAs for subsequent DGGE-analysis was performed using the primer sets GM5F (341F) with a GC clamp and 907R for bacteria (Muyzer et al. 1995), ARC344F-GC and 915R for archaea (Casamayor et al. 2000) as well as DSRp2060F-GC (Geets et al. 2006) and DSR4R (Wagner et al. 1998) targeting sulphate reducing bacteria, following the respective PCR protocols of the former authors. PCR products in an amount ranging from 300 to 500 ng was applied to DGGE as described by Schäfer & Muyzer (2001), using the D-Code system (Bio-Rad Laboratories, CA). Electrophoresis was performed with 6 % polyacrylamide gels (ratio of acrylamide to bisacrylamide, 40:1) submerged in 0.75 x TAE buffer (40 mM Tris, 40 mM acetate acid, 1 mM EDTA, pH 7.5) at 60°C and run for 16 hrs at 100 V in a linear 20 to 70 % denaturant gradient. Stained with ethidium bromide (0.5 g/ml), individual bands were excised and re-suspended overnight at 4°C in 50 µl of Milli-Q water. After re-amplification with the original primer sets, PCR products were purified via the Qiaquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) prior to direct sequencing by using an ABI Prism 3100 genetic analyser (PE Applied Biosystems).

The partial 16S rRNA sequences were assembled and manually corrected using the BioEdit sequence alignment editor (Hall 1999), and further compared to the Gen-Bank database by the BLAST analysis tools of the National Centre for Biotechnology Information (Altschul et al. 1990) in order to determine their phylogenetic affiliation and to identify their closest phylogenetic relatives.

The deduced, partial dsrB sequences of the environmental samples were implemented into the persisting dsrAB alignment of SRP references strains, including all full-length Dsr sequences available from the public databases. The data sets were phylogenetically analysed with the tree inference methods included in the ARB software package (Ludwig et al. 2004) and the Phylogeny Inference Package Version (PHYLIP) 3.5c (Felsenstein 1993).

Results

Water chemistry

Water chemistry data are given in Tables 1 and 2B. Our three-years-monitoring of the pond water chemistry did not reveal any extensive seasonal variation or other fluctuation. Therefore, data given in Table 1 and 2B are mean values of five sampling time points. Chemical variations observed between the inlet, main pond and outlet are rather small (Table 1). The concentration of dissolved iron (FeII) at the inlet (1.6 mg/l) is almost completely consumed in the pond (outlet: 0.1 mg/l). Alkalinity drops from 184.8 mg/l to 168.6 mg/l as does Ca²⁺ from 374 mg/l to 348 mg/l. On the other hand, the concentrations of PO₄³⁻ (44.8 mg/l), NO₃⁻ (1.9 mg/l), and SO₄²⁻ (333.6 mg/l) increase to 50.4 mg/l, 11.0 mg/l, and 346.5 mg/l, respectively. The oxygen (O₂) concentration in the water column varies between 1.2–4.8 mg/l (37–150 µmol/l), but closer to the mat surfaces the O₂ concentration rises from the 0.7 mg (21.1 mmol/l) at the inlet to 1.9 mg (58.0 mmol/l) at the outlet. Oxygen profiles starting at the water surface into the ochre mats (at 1 cm water depth) show a clear decrease in oxygen concentration (Fig. 3A). The decline depends on the density of the ochre mat. The fluffy nature of the mats is clear from the intrusion of water with a higher O₂ content to deeper parts in the mats. The oxygen consumption alters between the different layer of the mat and is much slower in the upper fluffy part than in the denser deeper parts. O₂ and pH profiles down into the white and black mat show a roughly stable water column above these mats, and a sharp drop in O₂ concentration at the mat surface (7 cm water depth). Concurrently the H₂S concentration rises significantly and the pH is shifted by 0.1 (Fig. 3B).
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**Microbial mat structure and community**

The different mat types differ significantly in their content of organic matter. The ochre mat contains 5.1 to 5.8 % C\textsubscript{org} (organic carbon), the black mat 15.4 % C\textsubscript{org} and the white mat 17.6 % C\textsubscript{org}.

Whereas the ochre mat consists mainly of iron oxyhydroxide impregnated stalks produced by iron oxidizing bacteria (IOB), numerous bacterial cells and EPS structures are visible in the black and white mat (SEM images, Figs. 4, 5). CLSM images from the lectine-stained ochre mat show a diffuse network of mineralized stalks without a clear relation to the stalk-producing cells (Fig. 6A). Some stalk fragments contained a non-encrusted protein band of unclear function (Fig. 6B).

The community compositions of the microbial mat at the inlet, outlet and in the main pond, respectively, were analysed by FISH, 16S rRNA gene analyses, and by 454 pyrosequencing. 454 pyrosequencing of the ochre mat resulted in 6500 sequences per sample, allowing a good overview of the large diversity of iron-oxidizing (depositing) bacteria. Among the identified genera were *Gallionella, Sideroxydans, Crenothrix, Marinobacter, Acidithiobacillus, Thiobacillus, Acidovorax*, a large diversity of Hyphomicrobiaceae including *Pedomicrobiurn, Filomicrobiurn*, and the recently isolated marine iron oxidizing bacterium *Mariprofundus* (Emerson et al. 2007) that dominated this environment. Although an initial clone library (data not shown) several sequences were related to known *Leptothrix* sequences none could be detected in the 454 sequences.

In comparision to sequences published in the International Nucleotide Sequence Database GenBank (in the following, accession codes are put in parentheses), sequences obtained by DGGGE band excision and re-amplification inter alia showed high similarities to two alpha-proteobacteria: a mucus bacterium (AY654810.2) and *Citreicella thiooxidans* (AY639887.1), a lithoheterotrophic sulphur-oxidizing bacterium, which had been detected at first in the Black Sea (Sorokin et al. 2005). The ochre microbial mats also harboured the beta-proteobacterium *Nitrosonomas* sp. (AB113603.2, AJ275891.2) and *Thioautota* sp.

**Table 2A:** Major cation concentrations in the microbial mats. The thick ochre mats were analysed in three different sections (each 7cm), in order to check for potential stratifications.

<table>
<thead>
<tr>
<th>y/kg</th>
<th>White mat</th>
<th>Black mat</th>
<th>Ochre core top</th>
<th>Ochre core middle</th>
<th>Ochre core bottom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>2.469</td>
<td>6.200</td>
<td>0.025</td>
<td>0.027</td>
<td>0.063</td>
</tr>
<tr>
<td>Ca</td>
<td>29.5</td>
<td>56.3</td>
<td>13.9</td>
<td>16.1</td>
<td>13.4</td>
</tr>
<tr>
<td>Cr</td>
<td>0.024</td>
<td>0.016</td>
<td>0.002</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>Fe</td>
<td>60.3</td>
<td>78.7</td>
<td>150.3</td>
<td>154.0</td>
<td>143.4</td>
</tr>
<tr>
<td>K</td>
<td>1.66</td>
<td>2.35</td>
<td>0.137</td>
<td>0.204</td>
<td>0.155</td>
</tr>
<tr>
<td>Mg</td>
<td>7.85</td>
<td>10.30</td>
<td>1.19</td>
<td>1.42</td>
<td>1.11</td>
</tr>
<tr>
<td>Mn</td>
<td>0.295</td>
<td>0.469</td>
<td>4.17</td>
<td>6.25</td>
<td>5.60</td>
</tr>
<tr>
<td>Na</td>
<td>53.6</td>
<td>30.8</td>
<td>5.46</td>
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<td>4.46</td>
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<tr>
<td>Sr</td>
<td>0.326</td>
<td>0.368</td>
<td>0.473</td>
<td>0.567</td>
<td>0.489</td>
</tr>
<tr>
<td>Ti</td>
<td>0.678</td>
<td>1.629</td>
<td>0.003</td>
<td>0.004</td>
<td>0.010</td>
</tr>
<tr>
<td>Si</td>
<td>7.05</td>
<td>13.71</td>
<td>16.10</td>
<td>18.42</td>
<td>17.77</td>
</tr>
</tbody>
</table>

**Fig. 6:** Laser scanning microscopy (CLSM) of an ochre microbial mat. Fluorescence lectin-binding analysis was performed using nucleic acid and protein-specific fluorochromes (green) as well as the D-glucose and D-mannose staining lectin (red). Mineral precipitates are visible as white reflections. In the mat aggregate, a clear recognition of cells and their relation to appending stalks was impossible (A). In a partly mineral impregnated stalk fragment a smooth protein end was detected (B).
Heim et al. (AJ632077.2), a sulphur-oxidizing delta-proteobacterium. The sulphate-reducing bacteria, *Desulfolobus propionicus* (AY953411.1, AY354098.1, AY354099.1, AY953400.1, DQ250787.1, AY953399.1), *Desulfolobus rhaediformis* (AB263181.1, DQ250778.1), *Desulfitobacterium autotrophicum* (AB263151.1) and *Desulfotalea psychrophila* (AY354094.1) were found in all mat types (white, black, and ochre). DGGE-derived sequences with high similarity to *Methylocalybos* sp (EF212354.1, AY424847.1, AY550695.1) were found in both ochre mat consortia connected to in- and outlet, as well as in white and black mats. Only two out of six archaeal species present in the ochre mat types could be blasted (NCBI, Blastx) yet, one with an uncultured Crenarchaeote (AF201358.1) and one with a ferromanganese micronodule archeon (AF293019).

**Microbial mat chemistry**

Microscopy and LA-ICP-MS were used to investigate TREE fractionations and accumulations within the different microbial mats. A distinctive TREE pattern of each mat type, if existing, can potentially serve as an inorganic biosignature for iron oxidizing, sulphate reducing bacteria and sulphur oxidizing bacteria (IOB, SRB, SOX) respectively. Major and trace element contents of the microbial mats are given in Tables 2A and 2B. In the ochre mat, Fe, Mn, and Si are considerably more abundant than in the white and black mats. These elements seem to be distributed evenly within the ochre mat as our analyses did not reveal any element accumulation in a particular zone of the mat profile (Table 2A; see also Fig. 2).

On the other hand, in the white and black mats, alkaline and alkaline earth metals (Ca, K, Mg, Na) and the elements Al, Cr and Ti are significantly enriched as compared to the ochre mat (Table 2A). In all microbial mat types, REE were enriched up to 10^4-fold compared to the pond water (Table 2B). Whereas some transition metals (Sc, V, Cr, Co) do not show significant accumulation in any of the microbial mats, other transition metals (Cu, Zn), as well as the elements Sc, As, Rh, Cs, Cd, Mo, Bi are highly enriched in the black and white mat (Tables 2A-B).

When normalising the observed element accumulation patterns on the pond water, it is possible to distinguish individual fractionation behaviours of the different mat types. Fig. 9 shows the accumulation of individual elements in order of increasing enrichment.

**Fig. 7:** Laser scanning microscopy coupled with fluorescent in situ hybridization (FISH) applying probes Beta42_og (beta-proteobacteria, labeled with Oregon green) and DSS658(cy3) (*Desulfosarcina/Desulfococcus*) on white consortia (A); Beta42(cy3) and SRB385(og) (sulfate reducing bacteria) applied to the black mat (B, C) from the main pond (abbreviations for fluorochromes cyanine 3 and Oregon green in parentheses).
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Fig. 8: Fitch-Margoliash tree (Consensus) of re-amplified DGGE-bands based on dsrB-derived PCR-products, indicating the encoding gene for the dissimilatory sulfite reductase. Areas indicate similarities to species matched by BLASTx (NCBI).
Comparing the black and white mat, they show a similar fractionation pattern, but rather contrasting to the ochre mat. The white and black mats show a generally higher enrichment of most of the trace elements except for the REE. The highest REE accumulation rates were observed in the ochre mat. Furthermore, the even fractionation pattern shows that the REE distribution of the ochre mat mirrors quite exactly the major water chemistry, but with an enrichment factor of about $10^4$. The white and black mats show a preferential enrichment of the light REE (La-Sm) over the heavy REE (Gd-Lu).

### Discussion

The water chemistry of the pond remained quite stable over the three years, and no seasonal variations were detected. The interaction between the microbial mats and the pond water seem to be rather stable. A remarkable impact of the microbial mat activity on the pond fluid is the maintenance of hypoxic conditions close to the mats (Table 1), even though the large pond surface is exposed to a “normal” atmosphere. Although the oxygen concentration of the pond water is significantly higher close to the pond surface, the microbial community of the ochre mat seems to be able to maintain favouring conditions to oxidize Fe(II). Keeping the O$_2$ concentration below 5 mg/l (ca. 150 µmol/l) in the main pond water enables a half life time of Fe(II) of several minutes (Emerson et al. 2010 and references therein). These conditions still allow iron oxidizing microbial communities to thrive and form thick microbial mats all over the pond.

The pyrosequencing results indicate that *Gallionella* sp. is not as abundant in the ochre mat as it was anticipated from previous reports (Pedersen & Hallbeck 1985; Hallbeck et al. 1993; Pedersen et al. 1996; Anderson & Pederson 2003). Rather, *Mariprofundus ferrooxidans* was found to be the dominating stalk-producing IOB in the studied pond. Although a visual differentiation through comparison of the order and structures of the stalks between certain *Gallionella* and *Mariprofundus* species is possible (Comolli et al. 2011), such differences where not observed in our samples. Both *G. ferruginea* and *M. ferrooxidans* are able to cast off their old, mineral-covered stalks and replace them by fresh ones (Cholodny 1926; Emerson et al. 2007). Due to the effective production of iron oxyhydroxides and the permanent replacement of heavily encrusted stalks, the ratio of mineral precipitates to organic matter is extremely high in these IOB-dominated mats.

The TREE accumulation in the ochre mat shows a signature, conserving mostly the TREE pattern of the feeder fluid, especially theREE (Fig. 9) with a constant $10^4$ enrichment. The iron oxidizing bacteria *Mariprofundus* sp. and *G. ferruginea* are able to control the iron mineralization process to some degree (Saini & Chan 2012). This would allow a localized distribution of the biotic and abiotic minerals formed. Although the stalk surface is smooth and pristine during the initial stage of formation, with increasing distance and age, the stalk becomes more and more coated with lepidocrocite (FeOOH) and two-line ferrihydrite (Chan et al. 2011).

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The accumulation rates of REE, Be, Y, Zr, Nb, Hf and W in the ochre mat were as high as those of Fe (Tables 2A-B) indicating a co-precipitation with the iron oxyhydroxides. This strong metal sorption capacity of iron oxyhydroxides is also widely used in technical applications and remediation activities (de Carlo et al. 1998; Bau 1999; Cornell & Schwertmann 2004; Michel et al. 2007).
However, not only the microbial iron oxyhydroxides, but also the EPS-composed stalks play an important role in TREE precipitation as they offer large reactive surfaces for the biosorption of metals. Metals with a higher positive charge tend to show enhanced biosorption (Haffnerburg et al. 2007). This may also explain the higher enrichments observed for the 3- and 4-valent elements Si, Mn, Y, Zr, Hf, U, and W compared to lower valent elements like Ca, K, Mg, Na, Cs, Rb and Li. However, although a preferential uptake of specific elements was not observed in the IOB dominated ochre mat, the general enrichment pattern seems to reflect the environmental conditions throughout the activity of the microbial mat.

According to DGGE as well as FISH results, the black and white mats seem to harbour similar community members mainly involved in sulphur oxidation, autotrophic denitrification, aerobic methylotrophy and sulphate reduction, which have been shown to build layers of dense aggregates (Fig. 6). Sulphur compounds are both used by SRB and autotrophic denitrifiers such as Thiobacillus denitrificans as electron donor, coupling the oxidation of inorganic sulphur compounds (such as hydrogen sulphide and thiosulphate) to the reduction of oxidized nitrogen compounds (such as nitrate, nitrite) to dinitrogen. This facultative anaerobic beta-proteobacterium was found also to couple the anaerobic oxidation of Fe(II) to denitrification, utilizing poorly-soluble minerals containing reduced iron and/or sulphur, such as pyrite (FeS₂) and FeS (Straub et al. 1996).

Overall, sulphate reducers are found in all type of mats presented here, while not all of them exhibited an active dissimilatory sulphate reduction; such, most sequences obtained by DGGE on the base of functional genes such as dsrB (Fig. 8) fall within the Desulfobacterium sp., Desulfovibrio sp. and Desulfomicrobium sp. clusters. However, any similarity to Desulfovibrio sp. might overlap with Desulfocapna sp., which was not included in this analysis. Despite their visual appearance, the microbial communities of the black and the white mat were rather similar, harbouring a significant amount of SRB, closely aggregated with beta-proteobacteria such as Thiobacillus denitrificans (Fig. 7). This is in good agreement with H₂S profiles (Fig. 3), in the white and black mat, showing a high concentration above the black mat and decreasing in the white mat. Although SEM analyses indicate the presence of filamentous, sulphur-oxidizing bacteria (Fig. 5), no respective sequences could be retrieved from DGGE band sequencing. In general, fingerprinting analyses need always careful interpretation, including the evaluation of bias of PCR primer-based strategies (Mahmood et al. 2006) and the fact of potentially poor amplicon separation by DGGE and re-amplification (Nikolausz et al. 2005). Consequently, the total of sequences obtained by DGGE band excision may not represent all members of the complete microbial community in the pond.

Although a great similarity between the microbial communities at the in- and outflow were detected the localized occurrence of specialized mat types in the pond argues for different controlling factors for the microbial mat formation. The substrate could be such a controlling factor. Indeed, the analysis of rock samples showed that at the growth location of the black and white mats, the surrounding grano-dioritic host rock was covered with concrete. Apparently, the presence of concrete created a micro-environment within the pond that favoured the presence of SRB and SOX communities. Concretes and cements commonly contain considerable amounts of either gypsum or sulphate rich fly ash (e.g., Iglesias et al. 1999; Guo & Shi 2008; Camarini & De Miltio 2011). Thus, the concrete may serve as a likely sulphate source for the microbial communities.

The high accumulation of Cu, Zn, Se, As, Rh, Cs, Cd, Mo, Bi in the black mat can be mainly attributed to the activity of SRB resulting in the production and precipita-
tion of iron sulphide minerals (Huerta-Diaz & Morse 1992; Watson et al. 1995; Beech & Cheung, 1995; Neumann et al. 2006). Studies showed that accumulations of TREE like Co, Cu, Mn and Ni with pyrite (FeS2) are linear to the amount of pyrite produced, due to co-precipitation of these trace elements with biogenic pyrite (Huerta-Diaz & Morse 1992). Therefore, the TREE fractionation pattern observed in the black was related to the dominating SRB in this mat. Further comparison to TREE fractionation in SRB-derived iron sulphides is difficult due to sparse reference literature. In most contemporary palaeontological studies, the morphological occurrence (e.g., pyrite framboids) or the fractionation of iron and sulphur isotopes is attributed to the activity of sulphate reducing prokaryotes (a.o., Beard et al. 1999; Icetpin et al. 2004; Shen & Buik 2004; Fortin & Langley 2005; Watson et al. 2009; Canfield et al. 2010; Cavalazzi et al. 2012). However, a systematic evaluation of TREE fractionation in biogenic pyrite is necessary to allow a further differentiation from hydrothermal pyrite and its validation as a biotically related biosignature. As the white mat seem to consist of several dominating communities (sulphate reducing, sulphur oxidizing and methanotrophic bacteria) the TREE pattern of these trace elements with biogenic pyrite (Huerta-Diaz & Morse 1992). Therefore, the TREE fractionation pattern of iron sulphide minerals formed pyrite within this mat. These observations point at a potential utility of this fraction pattern as a distinctive biosignature, but a further validation and comparison with other microbial mats and biotically and abiotically formed pyrites is necessary.

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