Cyanobacteria and Diatoms in Biofilms of Two Karstic Streams in Germany and Changes of Their Communities Along Calcite Saturation Gradients

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Biofilms microscopically dominated by cyanobacteria and diatoms of two CO2 degassing karst-water creeks in Germany were investigated for their diversities along a gradient of calcification using SSU rRNA gene cloning and sequencing from environmental samples. The recovered totals of 731/413 cyanobacteria/diatom clones were grouped at 97/98% similarity levels into 28/29 molecular OTUs widely spread over their corresponding sequence phylogenies forming mostly monophyletic subclades. Sequence comparisons with named reference strains from NCBI/GenBank as well as newly determined references from the SAG culture collection left about half of the cyanobacteria OTUs still unidentified. Most of the diatom OTUs could be identified at least at the generic level. To improve identification also cultures of cyanobacteria and diatoms were established that allowed even species identification of some diatoms, but also revealed additional cyanobacteria hard to identify which were not recovered in the clone libraries. A significant correlation of the relative OTU abundances in clone libraries with values of SIcalcite was found and, therefore, redundancy analysis distinguished highly calcified sites far from the spring from those less calcified closer to the spring. The noncalcified spring sites were clearly distinct from all other sites by the presence of four cyanobacteria OTUs exclusively retrieved and that no diatoms could be recovered from there. Four cyanobacteria and three diatom OTUs were recovered whose increasing relative abundance per clone library was correlated with increasing calcification. This may indicate that not only cyanobacteria, but also diatoms are more directly involved in the biogenic impact on tufa formation than assumed previously.

Keywords: Cyanobacteria, Diatoms, karst-water stromatolites, micro-algal community structure, molecular operational taxonomic unit (OTU), small subunit ribosomal Redundancy Analysis (RDA), RNA (SSU rRNA)

Introduction

Epilithic biofilms are frequently found on the surface of stromatolites in karst-water creeks (Figure 1). In the photic zone of such shallow-water environments stromatolites are often formed by these thin (<100 μm) biofilms. Recently, photosynthesis and respiration at the biofilm surface were found to cause precipitation and dissolution of carbonate (Bissett et al. 2008a, b) due to the photosynthetic and respiratory activity. In addition extracellular polymeric substances (EPS) associated with the biofilm may contribute to calcification (see Bissett et al. 2008a, b). Periods of precipitation and dissolution of carbonate due to changing appropriate environmental factors (Bissett et al. 2008b) may lead to the formation of macroscopically laminated benthic microbial deposits, called tufa stromatolites (Riding 1991).

Calcifying biofilms have contributed significantly to the formation of carbonate sediments throughout earth history (e.g., Bissett et al. 2008b; Riding 1991). Previous studies on calcification processes associated with those biofilms have
been conducted at two exemplar karst-water creeks in Germany, the Westerhöfer Bach (Figure 1) and the Deinschwanger Bach (e.g., Arp et al. 2001; Arp et al. 2010; Bissett et al. 2008a; Shiraishi et al. 2008a,b; Zippel and Neu 2010). Those calcifying biofilms on tufa stromatolites in both creeks are commonly dominated by diatoms and cyanobacteria producing significant amounts of EPS (Arp et al. 2001; Golubić 1976, Golubić et al. 2008, Janssen et al. 1999; Merz 1992; Merz-Preiß and Riding 1999, Pentecost 2005; Pentecost and Riding 1986; Zippel et al. 2010).

Earlier investigations considered calcite precipitation in tufa-forming systems as physicochemically driven via CO2 degassing (e.g., Chaletz and Folk 1984; Herman and Lorah 1987; Merz-Preiss and Riding 1999; Usdowski et al. 1979), with the biological impact restricted to the provision of nucleation site at exopolymers (e.g., Emeis et al. 1987). Later microsensor studies demonstrated that calcification in biofilm-covered areas is controlled by the photosynthetic CO2 removal of phototrophic microorganisms (Bissett et al. 2008a, b; Shiraishi et al. 2008a, b). The high abundance and

Fig. 1. A high calcified sampling site at the Westerhöfer creek and an example of a stromatolite (inset) covered with biofilms investigated in this study.
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diversity of cyanobacteria and diatoms in tufa biofilms have already been recognized more than a hundred years ago by Reichelt (1899) and have been reported continuously since that time (Couradeau et al. 2013; Dittrich and Sibler 2010; Freytet and Verrecchia 1998; Grüninger 1965; Kamennaya et al. 2012; Pia 1934; Pepe-Ranney et al. 2012; Plenković-Moraj et al. 2002; Reichardt 1995; Rott 1994).

However, apart from preliminary molecular analyses (e.g., Brinkmann et al. 2007a, b), cyanobacteria and diatom communities of calcareous microbialites were identified by morphology only, i.e., direct microscopy of biofilms covering tufa (e.g., Arp et al. 2001, 2010; Freytet and Verrecchia 1998; Plenković-Moraj et al. 2002). Traditional identification of these microorganisms by morphology in situ is difficult and often remains too ambiguous to recover the microbiotic taxa along various sampling sites with certainty. Recently, a considerable role of the photosynthetic activity in the calcification processes associated with the tufa stromatolites has been found (Bissett et al. 2008a, b; Shiraiishi et al. 2008a, b, 2010). Furthermore, Zippel and Neu (2010) characterized glycoconjugates of extracellular polymeric substances in tufa-associated biofilms by using fluorescence lectin-binding analysis. Hodač et al. (2014, this issue) investigated the diversity of microscopic green algae (Chlorophyta) in WB and DB. To close a gap of knowledge, the present study particularly employs a culture-independent approach to assess the diversities of the most abundant algal groups involved in formation of the biofilms, i.e., cyanobacteria and diatoms.

Materials and Methods

Study Area and Sampling

The study sites were two karst-water creeks in Germany, the Westerhöfer Bach (WB) and the Deinschwanger Bach (DB), located at the western margin of the Franconian Alb (49°23'N, 11°28'E). Both sites have been used for previous studies on calcification and are described in more detail in Arp et al. (2001, 2010) and Usdowski et al. (1979). Both sites show active laminated stromatolite formation of up to 0.18 mm per year (Bissett et al. 2008a). Briefly, the creek water of the Westerhöfer Bach provides comparatively high Mg²⁺ and SO₄²⁻ concentrations.

Biofilms from five sites of an approximately 330 m long section, i.e., from the spring site (aquifer; WBS) downstream including one low-calcified site (WBS2) and three high calcified sites further downstream were studied (WB3, WB4 and WB5; Tables 1, 2). The Deinschwanger Bach (DB) is fed by three main springs and a number of side springs with the water high in NO₃⁻ concentrations due to agricultural run-off. Biofilm samples were taken from one of the spring sites (DBS), one low calcified site close to the DBS spring site (DB9) and three sites with high SIcalc valeurs downstream (DB1, DB3 and DB6; Tables 1, 2).

A range of hydrochemical parameters have recently been measured at these sites and tables with the measured values are either available from Arp et al. (2010) or from G. Arp upon request. The DB biofilm samples were collected in autumn (October 5, 2005; October 9, 2006) and those from WB in summer and autumn (June 8, 2005; November 8, 2006). Additional samples from the WB were collected in 2010 at three different dates, March 5, May 26 and August 4. Biofilms were scratched from the surfaces of rocks which were submerged by creek water using sterile scalps, transferred into sterile 50 ml Falcon tubes (Sarstedt, Nümbrecht, Germany) and cooled until further processing in the laboratory which was 1–5 days after sampling.
Table 1. The four groups of cyanobacterial OTUs recovered from tufa-forming biofilms, their distribution on the sampling sites of the two creeks, DB and WB, and their relative abundances (percentages) per clone library.

| OTU names | Culture | No. of OTUs per site |
|-----------|---------|----------------------|
| Tychonema-relatives2 (OTU 7) (Identified A) | — | 1 43 24 2 21 7 32 6 |
| Tychonema-relatives4 (OTU 5) (Identified A) | — | 1 6 27 6 21 21 6 |
| Tychonema-relatives1 (OTU 6) K22 = SAG 2388 | — | 37 3 |
| Tychonema-relatives3 (OTU 4) (Identified B) | — | 9 |
| Leptolyngbya cf. foveolarum (OTU 28) AH36 = SAG 2413 | — | 9 21 |
| Unidentified 2 (OTU 2) (Identified E) | — | 2 |
| Limnothrix cf. redekei (OTU 121) AH21, AH14 | — | 3 |
| Getiferinema carotinum (OTU 124) | — | 3 |
| Wilmottia murrayi (OTU 9) | — | 63 |
| Nostoc sp.1 (OTU 16) AH38 = SAG 2414 | — | 3 |
| Nostoc sp.2 (OTU 15) AH02 = SAG 2409 | — | 21 3 4 |
| Synechococcus sp. (OTU 56) RK4 = SAG 2387 | — | 3 1 14 9 |
| Chamaesiphon spp. (OTU 33) (= Chamaesiphon sp.) | — | |
| Unidentified 5 (OTU 55) | — | |
| Unidentified 6 (OTU 132) | — | 18 |
| Pleurocapsa-like (OTU 20) | — | 8 |
| Chamaesiphon sp.1 (OTU 35) | — | 7 |
| Chamaesiphon sp.2 (OTU 37) | — | 7 |
| Pseudanabaena sp. (OTU 52) | — | 12 2 |
| (= Pseudanabaena sp.) | — | |
| Tolypothrix sp. (OTU 14) AH48 | — | |
| Lyngbya aestuarii (OTU 48) | — | 3 |
| Phormidium priestleyi (OTU 32) | — | 3 |
| Unidentified 1 (OTU 10) | — | 5 |
| Unidentified 3 (OTU 147) | — | + |
| Unidentified 4 (OTU 161) | — | 3 |
| Unidentified C | — | + |
| Unidentified F | — | + |
| Synechococcales sp.1 (OTU 26) | — | 1 |
| Aphanothece sp. (OTU 104) AH24 = SAG 2412 | — | |
| Geminocystis sp. (OTU 138) AH05 = SAG 2410 | — | |
| Cyanobium sp. (OTU 125) AH25 | — | + |
| Leptolyngbya sp.2 (OTU 42) AH10 | — | + |
| (= Unidentified D) | — | |
| Leptolyngbya sp.1 (OTU 81) AH16 | — | + |

Note. A "-" indicates the presence of an OTU at a certain site but that no relative abundance was available for the OTU. The sampling sites are arranged from the spring sites (DBS and WBS) downstream with DB6 and WB5 the sites farthest from the respective spring site. Where cultures are available for an OTU, the corresponding strain number is given. Names in brackets are those from Arp et al. (2010).
Table 2. The three groups of diatom OTUs recovered from tufa-forming biofilms, their distribution on the sampling sites of the two creeks, DB and WB, and their relative abundances (percentages) per clone library

| OTU names                                                                 | Culture                   | No. of OTUs per site |
|---------------------------------------------------------------------------|---------------------------|----------------------|
| Navicula veneta/gregaria/cryptotenella/tripunctata/ sp.1 (OTU 28)         | WF11 (N. veneta)          | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Achnanthidium minutissimum sensu (OTU 14)                                 | RK6 (A. saprophilum)      | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Cymbellales sp.1 (OTU 41)                                                 |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Cymbellales sp.2 (OTU 20)                                                 |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Fistulifera saprophila (OTU 24)                                           | RK97                      | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Gyrosigma sp. (OTU 4)                                                     |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Nitzschia dissipata/palea/pusilla (OTU1)                                  | TPA1, QB21                | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Nitzschia fonticola (OTU 33)                                              | WB1B1                     | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Phaeodactylum -related (OTU 6)                                            |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Surirella sp. (OTU 27)                                                    |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Planothidium sp. (OTU 8)                                                  | RK12 (P. frequentissimum) | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Navicula sp.2 (OTU 30)                                                    |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Encyonema sp. (OTU 17)                                                    |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Navicula obtonga (OTU 103)                                                |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Navicula sp. (OTU 29)                                                     |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Navicula sp. (OTU 4)                                                      |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Gomphonema sp.A (OTU 101)                                                 |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Eolimna minima/Sellaphora cf. seminulum (OTU 10)                          | RK85 (E. minima), RK25 (S. seminulum) | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Mayanacea atomus (OTU 37)                                                 | RK88                      | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Unidentified 1 (OTU 9)                                                   |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Surirella brebissoni (OTU 26)                                             | WF5                       | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Diatoma temne (OTU 3)                                                    |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Staurosira construens (OTU 7)                                            |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |
| Ulnaria ulna (OTU 39)                                                    |                           | DBS: 0  DB9: 1  DB6: 6  DB3: 6  DB1: 6  WBS: 0  WB2: 1  WB3: 10  WB4: 9  WB5: 7 |

Note. A “+” indicates the presence of an OTU at a certain site but that no relative abundance was available for the OTU. The sampling sites are arranged from the spring sites (DBS and WBS) downstream with DB6 and WB5 the sites farthest from the respective spring site. Where cultures are available for an OTU, the corresponding strain number is given. Names in brackets are those from Arp et al. (2010).
DNA Extraction, PCR, Cloning and Sequencing

To extract DNA from biofilm material it was subjected to cell breakage by either shaking in the presence of an equal volume of acid washed glass beads (425–600 μm, Sigma, Taufkirchen, Germany) in a Minibeadbeater, (BioSpec, Bartlesville, USA) for about 30 seconds at 5000 rpm or ground into powder under liquid nitrogen in a mortar using micro pistils and then transferred into the same buffer. Lysis buffer P was taken from the Invisorb Spin Plant Mini DNA extraction kit (Stratagene, Berlin, Germany) which was further used according to manufacturer’s recommendations to complete the DNA extraction. The DNA was checked by agarose gel electrophoresis and then used for PCR amplifications. The primers PCR 1 and PCR 18 (Wilmotte et al. 1994) were used to amplify 16S rRNA genes from cyanobacteria.

For diatoms, the newly designed 5' and 3' primers D1NS1 (5'-CTAGTCTACGCTGTCCTC-3') and D1800R (5'-GTGGATCTCCCTGGAAGT-3') which both preferentially amplify 18S rRNA genes from Bacillariophyceae were employed. The 25 μl PCR reactions contained 0.5 μl concentrated DNA (for cyanobacteria) or 2.5 μl of 1:10 diluted DNA (for diatoms) and 1.25 U Taq DNA polymerase (cyanobacteria: HotStarTaq from Qiagen, Hilden, Germany; diatoms: Bioline, Luckenwalde, Germany) in the respective PCR buffer with each dNTP at 200 μM and at 2.5 mM MgCl2.

To minimize PCR bias and the presence of chimeric PCR products three to five parallel PCR reactions were prepared and then pooled. PCR amplification was conducted with initial denaturation for 15 min (cyanobacteria) or 5 min (diatoms) at 95°C followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 52°C for 30 s (cyanobacteria) or 51°C for 60 s (diatoms) and an extension at 72°C for 120 s in a Primus 96P thermocycler (MWG-Biotech, Ebersberg, Germany). A final elongation for 10 min (cyanobacteria) or 30 min (diatoms) at 72°C completed the PCR. After checking yield and size of the PCR products by agarose gel electrophoresis, three to five PCR reactions per sample were pooled, purified using the NucleoSpin-Extract-Kit (Macherey-Nagel, Düren, Germany) and eluted in 30 μl elution buffer as provided by the kit.

The purified PCR products from the environmental samples were processed for cloning, those obtained from cultures directly used for sequencing. For cloning, the PCR products were ligated into the TOPO® vector (TOPO TA Cloning Kit, Life Technologies, Karlsruhe, Germany) and cloned into competent E. coli cells (Top10, Life Technologies, Karlsruhe, Germany). For insert check, positive clones from the master plates (1.6% LB agar with Ampicillin 100 mg/ml and X-Gal 40 mg/ml) were directly used as template for PCR using M13 primers according to the amplification protocol given by the manufacturer. PCR products were checked for containing an insert of the expected size by agarose gel electrophoresis.

Clones containing an insert of the expected size were cultured overnight in LidBac reaction tubes (Qiagen, Hilden, Germany) filled with 1 ml Luria-Bertani (LB) medium containing Ampicillin (100 mg/ml). Plasmid DNA was extracted using the NucleoSpin-Plasmid-Kit (Macherey-Nagel, Düren, Germany) and directly used for sequencing. Genomic DNA from pure cultures was directly used for sequencing. Cycle sequencing was in a Flexigene thermocycler (Techne, Staffordshire, UK) with an initial denaturation of 1 min at 96°C, followed by 35 cycles of 45 s denaturation at 96°C, 45 s annealing at 50°C and 3 min extension at 60°C in a reaction volume of 10 μl containing 3 μl of plasmid DNA, 2 μl of sequencing primer (0.8 pmol/μL), 1 μl T-buffer (5x), 2 μl Terminator Ready Reaction Mix (BigDye Terminator v3.1 Cycle Sequencing Kit, ABI, Darmstadt, Germany), 2 μl HPLC H2O. Cycle sequencing of almost complete cyanobacteria 16S rRNA genes was performed with a set of primers as described in Wilmotte et al. (1994).

For almost complete diatom 18S rRNA genes a set of five standard primers, i.e. the two forward primers 34F (5'-GTCCTAAAAGATATAGGCGATC-3') and 1122F (5'-GGCCTGAACTTAAAGGAATTG-3') modified from primer 1125->1141 of Elwood et al. (1985) as well as three reverse primers, i.e., 370R (5'-AGGCTCCCTCTCGGAATCRAACC-3') modified from primer 366->322 of Elwood et al. (1985), 895R (Hallmann et al. 2013) and 1263R (5'-GAACGGCCATGCACCAACC-3'), which was modified from primer 1277->1262 of Elwood et al. (1985). The sequencing reactions were separated on an ABI Prism 3100 (Applied Biosystems, Foster City, USA) automated sequencer.

The sequences were edited, contigs assembled using the program SeqAssem (Hepperle 2004) and the consensus sequences exported into ARB (Ludwig et al. 2004) and could be used for reliable phylogenetic analysis. Sequences were examined for possible chimeric artifacts using the programs CHECK_CHIMERA (Maidak et al. 1999) and Bellerophon (Huber et al. 2004). Potential chimeras were excluded from further analysis. Representative sequences of cyanobacteria and diatoms reported in this study have been deposited in GenBank (Supplementary Table S1).

OTU Identification and Phylogenetic Analyses

High quality partial SSU rRNA gene sequences for each clone, i.e., at least 400 nucleotides (nts) long were aligned using the MAFFT (Katoh et al. 2002) web application (http://mafft.cbrc.jp/alignment/server) with “Strategy” set to “E-INS-I” for optimal accuracy. This resulted in alignment blocks of 731 cyanobacteria sequences 491 nts long and 431 diatom sequences 510 nts long, which were further processed in MOTHUR (Schloss et al. 2009) to group the sequences into operational taxonomic units, OTUs. In addition, the partial sequences were added to a core phylogeny constructed from databases of full length cyanobacteria and diatom SSU rRNA gene sequences in the ARB program (version 05.05.26, Ludwig et al. 2004, http://www.arb-home.de) using the parsimony interactive tool in ARB and assisted by online alignment tools found in SILVA (http://www.arb-silva.de/; Quast et al. 2013). Cut-offs 97% for cyanobacteria and 98% for diatom sequences were found appropriate to
represent most OTUs as monophyletic subclades in the SSU rRNA phylogenies in ARB (Marande et al. 2009; Romari and Vaulot 2004; Stock et al. 2012).

To identify a representative sequence from each OTU the “get.OTUrep” command in MOTHUR was used and the selected rRNA gene sequence clone was sequenced over its almost full length (> 1200 nts) to be further used in phylogenetic analyses. A total of 33 (cyanobacteria 9, diatoms 24) almost full SSU rRNA gene sequences were determined from the clone libraries (see Table S1). In cases where we failed to determine almost full SSU rRNA gene sequences for an OTU we used an almost full reference sequence from the NCBI GenBank detected using BLASTN (Altschul et al. 1990; http://www.ncbi.nlm.nih.gov/) or determined the corresponding sequences from a strain of the SAG culture collection (Friedl and Lorenz 2012) if member of the same OTU. Therefore, the four cyanobacteria strains Chamaesiphon polonius SAG 32.87, Nostoc sp. SAG 25.82, “Stanieria cyanosphaera” SAG 33.87 and Synechococcus elongatus SAG 89.79 were sequenced over their 16s rRNA gene sequences.

The newly determined almost full SSU rRNA gene sequences and representative partial sequences for those OTUs where no almost full sequences could be determined were deposited in GenBank under the accession numbers as in Table S1. For the phylogenetic analyses the almost full newly determined and available reference SSU rRNA gene sequences were aligned using the MAFFT web application as described above and the alignment corrected by eye using BioEdit (Hall 1999). The alignments were then further processed in Gblocks 0.91b (Castresana 2000; Talavera and Castresana 2007) to remove divergent and ambiguously aligned blocks of the alignments with parameters set as default except for the minimum length of a block which was set as 2 and allowed gap positions set as “with half.”

The final data sets used for the phylogenetic analyses had 93 sequences for the cyanobacteria and 106 sequences for the diatoms and were 1450 and 1590 sequence positions long. The GTR+I+I model of nucleotide substitution was found to fit best for both datasets and with parameters as from jModelTest (Darriba et al. 2012; Guindon and Gascuel 2003). Maximum Likelihood phylogenies with 100 bootstrap replicates were calculated using RAxML 7.0.4 (Stamatakis 2006). As additional statistical support for internal branches support posterior probabilities were taken from Bayesian phylogenies calculated with MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001) with four Markov chains and 3,000,000 generations sampling every 100 generations were used with the first 25% of the sampled trees discarded. Posterior probabilities were then calculated from two independent runs using the 50% majority-rule consensus of the kept trees.

**Cultures and Microscopy**

Enrichment cultures and cultures on agar-plates were established from biofilm material using five different media for cyanobacteria to initiate growth, i.e., BG11, BG11 without citrate, Z, Z 45/4, ES (http://www.uni-goettingen.de/de/184982.html), and autoclaved creek water. For diatoms, liquid MiEB12 and WC media were used (http://www.uni-goettingen.de/de/184982.html). An inoculation loop with biofilm was diluted in 2 mL sterile site water and vortexed. For establishing enrichment cultures of cyanobacteria, a 100 μL of the suspension was spread on six agar-plates (1.5%) of the described media and creek water. In addition, a 100 μL of the suspension was added to six Erlenmeyer flasks each containing 20 mL of each growth medium and creek water, respectively. For enrichment cultures of diatoms, 100 μL of the suspension was added to each 20 mL of MiEB12 and WC medium.

The agar plates and flasks were kept at 18°C under a light dark regime of 14-h:10-h hours and 20–50 μmol photons m⁻²·s⁻¹ from white fluorescent bulbs. Isolates of cyanobacteria and diatoms were then developed by striking a drop of liquid culture suspension on an agar plate and then after a few days incubation picking single colonies which were again stroked on agar plates; the process was repeated until unialgal cultures were obtained. For diatoms, the 1.6% agarized Bacillariophyceae medium (http://www.uni-goettingen.de/de/184982.html) was used for this process and further maintenance of the cultures. Microscopic observations of cultures were conducted using an Olympus BX60 microscope (Tokyo, Japan) with Nomarski DIC optics and an attached ColorView III camera (Soft Imaging System, Münster, Germany).

Micrographs were processed using Cell²D image software (Soft Imaging System, Münster, Germany). Scanning Electron Microscopy (SEM) of diatoms frustules was done from pure liquid cultures using the protocol of Krammer and Lange-Bertalot (1986) and viewed in a LEO 430 SEM scanning electron microscope (Oxford Instruments, Oxford, UK). For molecular phylogenetic analyses of the newly established isolates, DNA was extracted from cultures, 16S/18S rRNA genes amplified and sequenced as described for the biofilm material above. The new sequences were added to the rRNA gene sequence alignments and phylogenetically analyzed together with those sequences obtained from the culture-independent approach.

**Multivariate Statistical Analysis**

Relative abundances of cyanobacteria and diatom OTUs at the studied sampling sites (Tables 1, 2) were used as input for the multivariate statistical analyses. Canonical ordination techniques were used to study the relations between microhabitat hydrochemistry (Table 2 in Arp et al. 2010; Arp and Reimer unpubl.) and the changes in cyanobacteria and diatom communities. After lengths of main gradients in the OTU abundance data were detected using indirect ordination techniques, i.e., Principal Components Analysis (PCA) and Detrended Correspondence Analysis (DCA), the Redundancy analysis (RDA) (Hill 1979; Jaarsma et al. 2007; Pfender et al. 1988) was selected to obtain the best fit model for the correlation of OTU abundance and the measured environmental parameters.

For statistical support the Monte Carlo test with 2000 permutations under reduced model without covariables was used (Hope 1968). From the total number of eleven
hydrochemical parameters which were tested initially, only the \( S_{\text{calcite}} \) and \( p\text{CO}_2 \) revealed significant correlations with OTU abundances. Ecologically relevant environmental parameters, i.e., conductivity, \( \text{NO}_3^- \), \( \text{TA} \), \( \text{pH} \) and \( \text{Mg}^{2+} \), were tested for correlation with changes in species composition of cyanobacteria/diatom communities. However, apart from \( S_{\text{calcite}} \) and \( p\text{CO}_2 \), we did not recover any significant correlation. All statistical analyses were performed with PAST version 2.12 (Hammer et al. 2001) and CANOCO version 4.5 (Ter Braak and Smilauer 2002).

Results and Discussion

**A Culture-Independent Assessment of Cyanobacteria Diversity**

A total of 731 cyanobacteria clones were retrieved from the 16S rRNA gene libraries established for each of the 10 sampling sites of both karst-water creeks. A 97% sequence similarity threshold was selected for the de novo derived operational taxonomic units (OTUs) because this revealed mostly OTUs which were also supported as monophyletic lineages and subclades in the phylogenetic analyses (Figure 2). Twenty-eight cyanobacteria OTUs were recovered by the culture-independent approach (Table 1). They could be divided into three groups, i.e. those which were found frequently at two or more calcified sites, those exclusively found at the noncalcified spring sites of the creeks (DBS, WBS), and those retrieved only from a single site (Table 1). Five additional OTUs, not retrieved by the culture-independent approach, were recovered from cultures (see below; Table 1).

There were 15 frequently recovered OTUs and they were with high relative abundances per clone library (Table 1). Out of them the two most frequent OTUs at the calcified sites could not be identified unambiguously, *Tychonema*-relatives 2 and 4 (OTUs 7 and 5, Table 1; corresponding to lineages Unidentified A and *Tychonema* sp. of Arp et al. 2010). They were recovered from eight and seven calcifying sampling sites, respectively. Both OTUs represented a monophyletic subclade (OTU 5) and an independent lineage (OTU 7) within a well-supported clade, clade B, of the 16S rRNA gene phylogeny (Figure 2). OTU 5 as well as clade B also included representatives of other genera, i.e., *Microcoleus* and *Phormidium*. Two more frequently recovered OTUs were within clade B, i.e., OTU 4 *Tychonema*-relatives 3 (corresponding to lineage Unidentified B of Arp et al. 2010) and OTU 6 *Tychonema*-relatives 1.

Out of these four closer related OTUs only OTU 5 may actually correspond to the genus *Tychonema* Anagnostidis and Komárek because it also contains *Tychonema bourrellyi* Anagnostidis and Komárek strain CCAP 1459 /11B, which is the authentic strain of the type (Suda et al. 2002). Our clones, represented by the *Tychonema* sp. GQ324965 sequence (Arp et al. 2010) formed a well-supported monophyletic lineage with *T. bourrellyi* (Figure 2). The other three OTUs, OTU 4, OTU 6 and OTU 7, were distant from *T. bourrellyi* and, therefore, may not represent the genus *Tychonema*. Their genus assignments remained unclear because they were neither close to *Phormidium autumnale* nor *Microcoleus vaginatus* which were also within clade B.

The latter species was even paraphyletic with *P. autumnale* and the three *Tychonema*-relatives OTUs (Figure 2). The second most frequently retrieved OTUs, each recovered from a total of five sites, were OTU 28 *Leptolyngbya cf. foveolarum* and OTU 2 Unidentified 2 (= lineage Unidentified E of Arp et al. 2010), which were distantly related from each other and the most frequently occurring OTUs 5 and 7. OTU 28 also formed a monophyletic subclade, but was within clade C. OTU 2 represented a single lineage within clade E (Figure 2) and it was left unidentified, simply because no named closer relative was available. Two more OTUs recovered from both creeks at a total of three sites were OTU 132 Unidentified 6 and OTU 33 *Chamaesiphon* spp. (Table 1).

For OTU 132 no full 16S rRNA gene sequence could be established and, therefore, is not included in the phylogeny of Figure 2. Analyses using the ARB program, which allows the addition of partial 16S rRNA gene sequences to a pre-calculated phylogeny, revealed OTU 132 as member of another distant clade, clade G. The OTU left unidentified because no closer named relative was available within clade G. Similarly, also for OTU 33 no full 16S rRNA gene sequence could be established, but the ARB analyses showed it was a close relative of two identified strains, *C. polonicus* SAC 32.87 and *C. subglobosus* PCC 7430, and they were within another independent clade, clade F (Figure 2). OTU 124 *Geitlerinema carotinosum* and OTU 16 *Nostoc* sp.1 were recovered only from single sites in each of both studied creeks (Table 1).

The genus *Geitlerinema*, characteristic for standing and flowing freshwaters with alkaline character as well as brackish and saline waters (Margheri et al. 2003), has already been described also as a rare species in karst environments in Yucatán, Mexico (Tavera et al. 2013). In addition, *Geitlerinema* species were isolated from saline calcifying microbial mats of Christmas Island (Kiritimati) in 2013 (Brinkmann, unpublished). For OTU 124 only partial 16S rRNA gene sequence could be obtained and the analyses revealed it as a close relative with a reference strain assigned to *G. carotinum* (acc. no. AY423710). Analysis of full reference sequence we obtained for OTU 16 *Nostoc* sp.1 showed this OTU being closely related to an unidentified *Nostoc* strain (acc. no. HQ591523) within clade A (Figure 2).

OTU 9 *Wilmottia murrayi* was retrieved from three sites of DB, but not from WB (Table 1). Also for this OTU only partial sequences could be obtained and our analyses showed that this OTU is a close relative of a *Phormidium*-like reference strain from Antarctica, *W. murrayi* (acc. no. DQ493872). Conversely, two other OTUs, OTU 15 *Nostoc* sp.2 and OTU 56 *Synechococcus* sp., were retrieved from 3 and 4 calcified sites of the WB, but not from DB. The full reference sequences we obtained for both OTUs formed subclades with named reference strains, i.e., an unidentified *Nostoc* strain (acc. no. DQ185227) within clade A and an unidentified *Synechococcus* strain, PCC 7502 (acc. no. AF448080), within clade H of the 16S rRNA gene phylogeny (Figure 2). Finally, OTU 55 Unidentified 5 was retrieved only from two sites of WB (Table 1).
Fig. 2. Maximum likelihood (ML) phylogenetic analysis of molecular OTUs representing cyanobacteria, as inferred from 17 almost full 16S rRNA gene sequences determined for cyanobacteria from tufa biofilms of the two studied hard karst-water streams and 76 other reference sequences. Each recovered OTU is indicated by grey shading. Newly determined sequences are indicated by sequence names in bold. Thick lines mark internal branches which were resolved in both ML and Bayesian tree topologies. Numbers at branches are bootstrap values (left) and Bayesian posterior probabilities (right).
Again, no full 16S rRNA gene sequence could be established for this OTU, but ARB analyses of the partial sequences showed the OTU a member of clade G, but without any named closer relative (Figure 2). Similarly, also OTU 121 Limnothrix cf. redekei was retrieved from two sites of WB only. We established two full reference sequences for this OTU and they were closer related to a strain assigned to L. redekei (CCAP 1443/1, acc. no. AJ580007) within clade H than to an unidentified strain of Pseudanabaena (PCC 6903, acc. no. AB039017) which was also within the same OTU (Figure 2).

A second group of tufa cyanobacteria was formed by five OTUs exclusively retrieved from the noncalcified spring sites of both creeks (DBS, WBS; Table 1). For OTU 52 Pseudanabaena sp. and OTU 20 Pleurocapsa-like only partial sequences could be obtained and the ARB-analyses revealed both as close relatives to a named reference strains, Pseudanabaena sp. PCC 6802 (acc. no. AB039016) and SAG 33.87 (acc. no. KF417641). The latter strain has been assigned to Stanieria cyanoaphera, but strain SAG 33.87 was not a close relative with strain S. cyanoaphera PCC 7437, which may serve as a reference while no sequence for the type of this species is available (analyses not shown). Then strain SAG 33.87 likely has been misidentified. The other two spring site OTUs were OTU 35 Chamaesiphon sp. 1 and OTU 37 Chamaesiphon sp. 2 which both were distant from the two reference sequences available for the genus, C. polonicus SAG 32.87 (acc. No. KF417651) and C. subglobosus PCC 7430 (Acc. No. AY170472) as well as the sequences representing OTU 33 Chamaesiphon spp., but were within the same clade of the 16S rRNA gene phylogeny, clade F (Figure 2).

Ten OTUs were recovered by the culture-independent approach only at single sites and, interestingly, also only in low relative low abundances (<6%) per clone libraries. For OTU 48 Lyngbya aestuarii and OTU 32 Porphidium priestleyi only partial sequences could be obtained and they had rather short genetic distances to named reference strains, L. aestuarii UTEX LB 2515 (acc. no. HQ419208), two Porphidium-like references strains from Antarctica, P. priestleyi (acc. no. AY493585 and AY493586), and Pseudanabaena sp. PCC 6802 (acc. no. AB039016). Our full reference sequence of OTU 14 Tolypothrix sp. was within a monophyletic sub-clade of clade A which comprised of two strains representing the genus Tolypothrix, but also included a reference strain from Antarctica assigned to Coleodesmium (Figure 2).

Six more OTUs were left unidentified because no closer related named reference strains were available for them. Out of them only two OTUs, OTU 10 Unidentified 1 and Unidentified F (≡ lineage Unidentified F of Arp et al. 2010) were somehow related to OTUs retrieved frequently from both studied creeks, i.e., OTU 2 Unidentified 2 (Figure 2) and OTU 32 Porphidium priestleyi (not shown). The full reference sequences we obtained for OTU 26 Synecochoccales sp.1 and OTU 147 Unidentified 3 revealed closer relationships with unidentified environmental clones (acc. no. EF438248 and DQ269094). OTUs 161 Unidentified 4 and Unidentified C (≡ lineage Unidentified C of Arp et al. 2010) formed two independent lineages in the 16S rRNA gene phylogeny (Figure 2).

The majority of cyanobacteria OTUs recovered in our study by the culture-independent approach were assigned to genera which exhibit a filamentous morphology without heterocytes. They were members of the clades B, C and H and represented various other independent lineages of the 16S rRNA gene phylogeny (Figure 2). That filamentous nonheterocytous cyanobacteria are predominant in both studied karst-water creeks has already been observed earlier (Arp et al. 2001 for the DB) and has also recently been substantiated by Schneider et al. 2014 (this issue). The cyanobacteria lineages they recovered at DNA and RNA levels were affiliated with the Tychonema/Phormidium/Microcoleus clade (TPM; corresponding to our clade B and OTUs 9 W. murrayi and 48 L. aestuarii) and exhibited an average abundance of approximately 50% of the overall biofilm community.

Furthermore, our finding of numerous OTUs attributable to filamentous nonheterocytous cyanobacteria is in congruence with other studies on cyanobacteria in limestone caves in Greek and Argentinia (Ferrari et al. 2002; Lamprinou et al. 2012), in Pamukkale travertines in Turkey (Pentecost et al. 1997) or freshwater tufas of the Iberian Range (Beraldi-Campesi et al. 2012). Cyanobacteria with rather thin nonheterocytous filaments, representing the Leptolyngbya and Pseudanabaena morphotypes, have also been found dominating calcifying biofilms of the DB creek using resin-embedded hand part sections of the biofilms (Arp et al. 2001, Arp et al. 2010).

**A Culture-independent Assessment of Diatom Diversity**

Using a culture-independent cloning and sequencing approach a total of 413 clones representing diatoms (Bacillariophyceae, Stramenopiles) were recovered from 18S rRNA gene libraries (Table 2). For the diatom sequences, a 98% sequence similarity threshold was selected for the definition of OTUs because this revealed most OTUs as monophyletic lineages and subclades in the phylogenetic analyses (Figure 5). Twenty-nine diatom OTUs were recovered by the culture-independent approach (Table 2). They covered 17 raphid pennate genera, which represented a well-supported monophyletic clade in the phylogenetic analyses (Figure 5), congruent with other current diatom 18S rRNA gene phylogenies (e.g., Bruder 2006; Medlin and Kaczmarska 2004; Pniewski et al. 2010; Sims et al. 2006; Sorhannus 2007; Theriot et al. 2009).

In addition, three genera of araphid pennate diatoms were retrieved (Figure 5). We distinguished three groups of diatom OTUs, i.e., six OTUs which were recovered from four or more sampling sites, eleven OTUs found at two or three sampling sites and a group of twelve OTUs retrieved only at single sites (Table 2). In contrast to cyanobacteria, no diatoms could be recovered from the spring sites of both creeks (DBS, WBS; Tab. 2). In addition, in order to better identify certain diatom OTUs through employing SEM of their frustules, also a cultures were established (Table 2; see below).

OTU 28, consisting of at least five closely related Navicula species, i.e., _N. cryptotenella, N. gregaria, N. tripartata, N. veneta_ and an unidentified species ( _N. sp.1_), represented the
most frequent diatoms which was found at all calcified sites of both creeks except DB6 (Table 2). OTU 28 was not resolved as a monophyletic subclade in the 18S rRNA gene phylogeny. Its members formed five distinct lineages which were intermixed with other OTUs and species representing the genus *Navicula* (Figure 5). Monophyly of the genus, however, was well resolved. The second most frequently recovered OTU (from six calcified sites) was OTU 14 named *Achnanthidium minutissimum sensu lato* (Table 2).

The OTU comprised of two species, *A. minutissimum* and *A. saprophilum* (see below, cultures of cyanobacteria and diatoms). OTU 2, also found at six calcified sites, represented another species of *Achnanthidium*, but no closer named reference sequence was available. Both *Achnanthidium* OTUs formed a monophyletic clade in the 18S rRNA gene phylogeny (Figure 5). Three more frequent OTUs represented the genera *Gomphonema* (OTUs 21, 22) and *Pinnularia* (OTU 12). Interestingly, they seemed to have a preference towards the WB creek, i.e. OTU 21 *G. microps* and OTU 12 *P. rupestris* were retrieved from three sites of WB, but only a single site of DB. OTU 22 *Gomphonema* sp. B was even found at all calcified WB sites, but not a single time in the DB (Table 2).

The 11 diatom OTUs recovered at two or three sampling sites were assigned to the genera *Amphora*, *Fistulifera*, *Gyro-sigma*, *Navicula*, *Nitzschia*, *Planothidium* and *Surirella* (Table 2). Except for OTU 24 *Fistulifera saprophila* and OTU 33 *Nitzschia fonticola* which formed monophyletic subclades with named reference sequences in the 18S rRNA gene phylogeny (Figure 5), none of these OTUs could be identified down to species level due to the lack of appropriate reference sequences. OTU 1 did not form a monophyletic subclade, but consisted of two distinct lineages, representing *Nitzschia palea* and *N. pusilla*. Interestingly, a full clone sequence of OTU 6 formed a monophyletic subclade with a corresponding sequence from the marine *Phaeodactylum tricornutum*.

The monotypic genus *Phaeodactylum* occupies an independent position within pennate raphid diatoms in the 18S rRNA gene phylogenies (Figure 5; Bruder and Medlin 2008). Two OTUs could not be identified even at the genus level. OTU 41 Cymbellales sp.1 formed an independent lineage within the monophyletic Cymbellales clade. It was distant to the genera *Cymbella*, *Cymbopleura*, *Gomphonema* and *Encyonema*, which formed monophyletic subclades within the Cymbellales clade (Figure 5). For OTU 20 Cymbellales sp.2 only partial sequences could be determined. The ARB analyses showed that it was closer related to *Cymbopleura naviculiformis* (acc. no. AM501997) than to other members of the Cymbellales clade.

Diatoms that were recovered only from single calcified sites formed either OTUs or species of the same genera as already recovered more frequently, i.e., they represented four additional species/OTUs of *Navicula*, *Gomphonema* and *Surirella*, or represented additional genera recovered only at a single site, i.e., *Encyonema*, *Eolimma*, *Mayamae*, *Sellaphora*, and the araphid pennates, *Diatoma*, *Staurosira* and *Ulnaria*. One OTU (OTU 9 Unidentified 1) left unidentified even at the generic level, since no named closer neighbor was available (Figure 5) representing a monophyletic subclade together with the marine genus *Rossia* (EF 151968, Evans et al. 2007; Figure 5). Interestingly, the diatom OTUs we recovered only a single time were mostly from the WB (8 OTUs from WB, 3 from DB; Table 2).

**Cultures of Cyanobacteria and Diatoms**

Many cyanobacteria OTUs we retrieved from both studied creeks were left unidentified or their identification was ambiguous. Therefore, we attempted to culture cyanobacteria from WB sites to achieve an identification using morphological criteria for many OTUs. Despite all our attempts we succeeded in only 12 unialgal strains out of which seven could be accessioned by the SAG culture collection (Table 1). Six of the OTUs recovered by the culture-independent approach were also present in the cultures (Table 1). Five isolates were non-heterocytous filamentous or *Nostoc*-like cyanobacteria (Figures 3a-c, 3e and 3f) and one culture represented OTU 56 with a *Synechococcus*-like morphology (Figure 3d). Three of our isolates, AH36 (= SAG 2413), AH10, and AH16, exhibited morphological features similar as described for *P. foveolarum* (Figure 4d; see Komárek and Anagnostidis 2005).

Cyanobacteria attributed to *Leptolyngbya foveolarum* (syn. *Phormidium foveolarum*) as based on morphology were frequently encountered in both the DB and WB creek (Arp et al. 2001, Arp et al. 2010). Strain AH36 (OTU 28) was in fact phylogenetically very close to a strain assigned to *L. foveolarum* (PMC302.07; acc. no. GQ859653; Thomazeau et al. 2010), but no strain exists and has been sequenced that could be referred to as a “type” for the species. The two other strains with a *L. foveolarum*-similar morphology, AH10 and AH16, represented two OTUs unrelated to each other and were also rather distant from strain AH36. Strain AH16 (OTU 81 *Leptolyngbya* sp.1) was in clade G, and strain AH10 (OTU 42 *Leptolyngbya* sp.2) formed an independent lineage (Figure 2). Therefore, the same morphological features may be expressed in at least three distinct lineages of filamentous non-heterocytous cyanobacteria and the same morphotype may hide several phylogenetically distinct lineages.

Our isolates also recovered five OTUs which were not found by the culture-independent approach (Table 1). OTU 104 *Aphanthece* sp. (isolate AH24; Figure 4a) had just 4 and 8 nucleotide differences in its 16S rRNA gene sequence with the reference strains for *A. stagnina* (FR873836) and *A. microscopica* (FR873836, representing the type of the genus, Komárek et al. 2011), but 15 with the reference strain available for *A. sacrum* (AB116658 and AB111259). Therefore, our isolate may represent a species of *Aphanthece* not sequenced so far and not covered by the recent study of the genus by Komárek et al. (2011). A unicellular cyanobacterium assigned to *Aphanthece castagnei* was frequently observed in WB (Arp et al. 2010).

We assume it is represented by OTU 104 *Aphanthece* sp. which we retrieved only in culture. Identification of strain AH25 (Figure 4b) was ambiguous. It represented OTU 125 *Cyanobium* sp., which formed a monophyletic subclade of clade D together with a strain assigned to *Cyanobium* (acc.
no. AM710352) as well as strain SAG 89.79 assigned to Synechococcus (acc. no. KF417646; Figure 2). Strain AH05 representing OTU 138 Geminocystis sp. was characterized by vegetative cells with distinctly striated centroplasma (Figure 4c), rather similar to Geminocystis papuanica (Korelusová et al. 2009), widely oval cell shape with size (length/width) 6–8 μm/4 μm, the presence of binary cell division and cells often forming irregular colonies. It was a close relative with Geminocystis herdmani PCC 6308 (AB039001; Korelusová et al. 2009; Figure 2).

Comparison with the partial sequence available for G. papuanica (EF555569) revealed numerous differences. Therefore, we assume our isolate AH05 (= SAG 2410) to represent another species of Geminocystis not covered by DNA sequence analyses so far. For another lineage of tufa cyanobacteria which previously could not be identified, Unidentified D of Arp et al. (2010), isolate AH10 revealed a Leptolyngbya-like morphology (OTU 42 Leptolyngbya sp. 2). Isolate AH16 also exhibited a Leptolyngbya-like morphology (Figure 4d), but represented another OTU (OTU 81

Fig. 3. Morphology of cyanobacteria isolated from tufa-covering biofilms into culture and which were also recovered by the 16S rRNA gene cloning approach. Filamentous morphotypes (a), (b) and (c) without heterocytes, Tychonema -relatives 1 strain K22 (OTU 6) (a) and (b), Limnothrix cf. redekei strain AH21 (OTU 121) (c), coccal morphotype of Synechococcus sp. strain RKC4 (OTU 56) (d). Filamentous morphotypes (e) and (f) with heterocytes, Tolypothrix sp. strain AH48 (OTU 14) (e), Nostoc sp. -strain AH02 (OTU 15) (f). Scale bar 20 μm.
Leptolyngbya sp.1), which was not closer related to the former isolate (Figure 2).

To achieve a more precise species identification for several OTUs of diatoms which comprised several species each and to test the species assignments of molecular OTUs we attempted to establish cultured isolates of diatoms, i.e., for gaining supplementary characters from frustule morphology. Eleven isolates of diatoms were established from both creeks. Scanning electron microscopy (SEM) of the isolates identified Navicula veneta Kützing from OTU 28 (which comprised five different species; Figure 6a), Achnanthidium saprophilum (Kobayasi and Mayama) Round and L. Bukhtiyarova from OTU 14 (Figure 6b), Nitzschia fonticola (Grunow) Grunow (Figure 6c), Planothidium frequentissimum (Lange-Bert.) Lange-Bert. from OTU 8 (Figure 6d), and both, Eolimna minima (Grunow) Lange-Bert. (Figure 6e) and Sellaphora seminulum (Grunow) D.G. Mann, from the same OTU 10.

In other cases SEM of the isolates confirmed species assignments, i.e., Fistulifera saprophila (Lange-Bertalot and Bonik) Lange-Bertalot for OTU 24, Nitzschia fonticola (Grunow) Grunow for OTU 33, Mayamaea atomus (Kützing) Lange-Bertalot for OTU 37 and Surirella brebissoni Krammer and Lange-Bert. for OTU 26 (Figure 6f). Interestingly, the latter two species represented the only diatom OTUs, which were not recovered by the culture-independent approach as well. They may have escaped their molecular detection due to PCR-related biases (Stoeck et al. 2006).

**Cyanobacteria and Diatom Diversities in Relation to the Calcification Gradient Present in Both Creeks**

At the calcified sites both creeks were similar in their cyanobacteria and diatom diversities as determined from the total numbers of OTUs recovered by the culture-independent approach, although WB appeared a little more diverse. From DB total numbers of 17/20 different cyanobacteria/diatom OTUs were recovered, from WB the total numbers were 21/22 cyanobacteria/diatom OTUs. For WB the numbers of OTUs per calcified site were higher than those for DB, i.e., at WB maxima of 11/13 cyanobacteria/diatom OTUs were reached compared to 7/6 at DB. Furthermore at WB there were more OTUs which were exclusively recovered just from one of the two creeks, i.e., 2/3 cyanobacteria/diatom OTUs at WB compared to 1/1 cyanobacteria/diatom OTU at DB.

The total numbers of OTUs per site appeared not to be influenced by the gradient in calcification as seen from the increasing $SI_{calcite}$ values along the stream.
Fig. 5. Maximum likelihood (ML) phylogenetic analysis of molecular OTUs representing diatoms, as inferred from 35 almost full 16S rRNA gene sequences determined for diatoms from tufa biofilms of the two studied hard karst-water streams and 71 other reference sequences. Each recovered OTU is indicated by grey shading. Newly determined sequences are indicated by sequence names in bold. Thick lines mark internal branches which were resolved in both ML and Bayesian tree topologies. Numbers at branches are bootstrap values (left) and Bayesian posterior probabilities (right).
courses of both creeks. For the cyanobacteria, no clear trend in the variation of OTU numbers per site could be observed (Table 1). For diatoms, at DB the numbers of OTUs per calcified site was invariant, whereas at WB the numbers of OTUs per calcified site (10 and 13) were slightly higher at the lower calcified (WB2 and WB3) than at the higher calcified sites (9 and 7 at WB4 and WB5). However, when using the relative OTU abundances (percentages) per clone library from a certain site (presented in Tables 1 and 2) a statistically significant (p < 0.05) ordination of the sampling sites of both creeks was revealed using a linear method, i.e. Redundancy Analysis (RDA).

The ordination was based on the correlation between relative OTU abundance and values of SIcalcite (Figure 7). The two low calcified sampling sites close to the spring sites (with courses of both creeks. For the cyanobacteria, no clear trend in the variation of OTU numbers per site could be observed (Table 1). For diatoms, at DB the numbers of OTUs per calcified site was invariant, whereas at WB the numbers of OTUs per calcified site (10 and 13) were slightly higher at the lower calcified (WB2 and WB3) than at the higher calcified sites (9 and 7 at WB4 and WB5). However, when using the relative OTU abundances (percentages) per clone library from a certain site (presented in Tables 1 and 2) a statistically significant (p < 0.05) ordination of the sampling sites of both creeks was revealed using a linear method, i.e. Redundancy Analysis (RDA).

The ordination was based on the correlation between relative OTU abundance and values of SIcalcite (Figure 7). The two low calcified sampling sites close to the spring sites (with
similar $\text{SI}_{\text{calcite}}$ values clearly lower than those of the other sites; Tables 1 and 2), i.e. WB2 and DB9, were separated from the higher calcified sites (with higher $\text{SI}_{\text{calcite}}$ values). Among the latter sites two groups were discriminated, i.e., one with DB1, DB3 and DB6, and the other with WB3, WB4 and WB5. Although the DB sites were closely together, the WB sites were more distant with each other (Figure 7). The $\text{SI}_{\text{calcite}}$ values were lower in the three WB sites than in the three DB sites and $\text{SI}_{\text{calcite}}$ values had more variation among the three WB sites than those of the DB (Tables 1, 2).

At both the noncalcified spring sites, total numbers of recovered cyanobacteria OTUs was in about the same range as for the calcified sites, i.e., 6 and 4 cyanobacteria OTUs at DBS and WBS. However, the spring sites

![Fig. 7. Redundancy Analysis (RDA) to investigate possible correlations of relative abundance of cyanobacterial and diatom OTUs per clone library (sampling site) and calcification (expressed by saturation index $\text{SI}_{\text{calcite}}$). The first ordination axis (RDA 1) is statistically significant at the 0.05 level. Highlighted are those four cyanobacteria and three diatom OTUs for which a significant correlation of their presence and increasing values of the saturation index $\text{SI}_{\text{calcite}}$ was found. Names of cyanobacterial OTUs are underlined. The filled circles represent the highly (DB1, DB3, DB6; WB3, WB5) and low-calciﬁed (DB9, WB2) sampling sites. Fillings in black and grey reﬂect the recovered proportions of cyanobacteria and diatoms, respectively, at a sampling site.]

\[\text{SI}_{\text{calcite}}\]
exhibited significant differences compared to the calcified sites. There were no diatoms and three cyanobacteria OTUs exclusively found at the spring sites.

Using the relative abundances of OTUs per site the multivariate analysis depicted cyanobacteria and diatom OTUs for which their increasing abundance was correlated with increasing values of $\text{SI}_{\text{calcite}}$ (Figure 7). These were the cyanobacteria OTU 6 *Tychonema*-relatives 1, OTU 9 *Willemottia murrayi*, OTU 28 *Leptolyngbya cf. joveolarum* and OTU 124 *Geitlerinema* cf. *carotinobium* and the diatom OTUs, OTU 41 Cymbellales sp.1, OTU 28 *Navicula veneta/gregaria/cryptotenella/tripunctata/sp.1* (as *Navicula* spp. in Figure 7) and OTU 26 *Surirella* sp. We assume that these cyanobacteria and diatoms are also more directly involved in the calcification processes in both creeks than others recovered in our study. A cyanobacteria isolate representing OTU 6 *Tychonema*-relatives 1, strain K22 (= SAG 2388), has been found to actively influence calcification in an experimental study (Spitzer et al. 2014, this issue) and this finding is concordant with the biogenic impact in tufa formation of the natural biofilms from creek stone surfaces as revealed recently (Bissett et al. 2008a, b, Shiraishi et al. 2008a, b).

Interestingly, the diatom OTU 28 *Navicula* spp. was the most frequent diatom OTU; it was recovered from all calcified sites except DB6 and also with high relative abundances per site/clone library (Table 2). Also the diatom OTU 41 Cymbellales sp.1 occurred in particularly high relative abundances at two sites of DB (DB1 and DB3; Table 2). Therefore, it is tempting to assume that also certain diatom species may be involved in the biogenic calcite precipitation. However, so far diatoms have been regarded to occupy just a subordinate role in tufa stromatolite formation as compared to cyanobacteria. The exopolymeric diatom envelopes commonly remain uncalkified within the same biofilms (Arp et al. 2001), but for several diatom genera calcite precipitation around their stalks or at gelatinous sheaths has been reported and thus they seem to be involved in the precipitation of microcrystalline calcite (Winsborough and Golubić 2004). Close relatives of the OTU 41 Cymbellales sp.1 within the Cymbellales clade (Figure 5), i.e., species of *Gomphonema*, have been reported as producers of extracellular polysaccharides in porous calcareous stromatolitic deposits (Winsborough and Golubić 2004). Increased diatom abundances in the biofilms of DB have been observed during winter in DB and this may result in porous calcareous laminae with larger calcite crystals (Arp et al. 2001). Several of the diatom species recovered in our study have already been described as dominant members of benthic diatom assemblages on solid substrates in various other karstic streams with comparable pH and conductivity values. In particular, these were members of the genus *Navicula*, e.g., *N. gregaria* and *N. dissipata* (Chatháin and Harrington 2008; Sabater 1990).

Carbonate precipitation (calcification) may impose restricted exposure to light on the cyanobacteria and diatoms dwelling in the creek biofilms. Also the relative high CO$_2$ partial pressures in both creeks, particularly high at the spring sites, may represent an additional difficulty to photoautotrophic organisms, despite the essential role of CO$_2$ capture for building up carbohydrates. That increased CO$_2$ concentrations may lead to increased growth of microalgae is commonly observed in laboratory growth experiments, but from a certain CO$_2$ concentration level on the microalgal growth can be even suppressed (e.g. Mudimu et al. 2014, this issue).

Calcification (as expressed by $\text{SI}_{\text{calcite}}$) and $p$CO$_2$ are reciprocally linked, i.e. the sites with high $\text{SI}_{\text{calcite}}$ values are relatively low in $p$CO$_2$ and vice versa. The highly calcified sites of the WB (with low $p$CO$_2$) exhibited also the highest numbers of cyanobacteria OTUs, whereas the WB spring site (with high $p$CO$_2$) had the lowest cyanobacteria OTU number, but with certain OTUs found exclusively at these sites (Table 1). Enhanced $p$CO$_2$ may be more unfavorable to cyanobacteria than calcification. Similarly, all calcified sites of both creeks harbored rich diatom assemblages, but the spring sites (with high $p$CO$_2$) were found devoid of diatoms.

However, that the low-calcified site WB2 (with still relatively high $p$CO$_2$) was the site richest in diatoms cannot be explained only by the $\text{SI}_{\text{calcite}}$/pCO$_2$ ratios; additional other physico-chemical factors as well as light reaching the biofilms may influence species richness and taxonomic composition. Although RDA correlations between cyanobacteria/diatom OTU composition (as relative abundances) and various other physicochemical factors (e.g., pH, conductivity, $\text{NO}_3^-$, total acidity TA, $\text{Mg}^{2+}$; see Table 2 in Arp et al. 2010) were tested, only $\text{SI}_{\text{calcite}}$ and $p$CO$_2$ were found significant parameters to determine the cyanobacteria and diatom diversities.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.
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Jaarsma NG, Bergman M, Schulze FH, Vaate AB. 2007. Macro-invertebrates in a dynamic river environment: analysis of time series from artificial substrates, using a ‘white box’ network modeling method. Aquat Ecol 41:413–425.

Kamnennya NA, Ajo-Franklin CM, Northen T, Janson C. 2012. Cyanobacteria as biocatalysts for carbonate mineralization. Minerals 2:338–364.

Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucl Acids Res 30:3059–3066.

Komárek J, Anagnostidis K. 2005. Cyanoprokaryota, part 2. Oscillatoriae. In: Bäbel, W., Gärtringer, G., Krienitz, L. & Schagerl, M. (eds) Süßwasserflora von Mitteleuropa Band 19/2. Gustav Fischer, Jena, 1–759.

Komárek J. 2011. Recent changes (2008) in cyanobacteria taxonomy based on a combination of molecular background with phenotype and ecological consequences (genus and species concept). Hydrobiologia 639:245–259.

Komárek J, Kaštovský J, Jezberová, J. 2011. Phylogenetic and taxonomic nomenclature of the cyanobacteria genus Anaphothece Nägeli and Anathaceae (Komárek et Anagnostidis) comb. nova. European Journal of Phycolology 46:315–326.

Korelusová J, Kaštovský J, Komárek, J. 2009. Heterogeneity of the cyanobacteria genus Synechocystis and description of a new genus, Geminocystis. Journal of Phycolology 45:928–937.

Krammer K, Lange-Bertalot H. 1986. Bacillariophyceae. Teil: Naviculaceae in Ettl H, Gerloff J, Heyning H and Mollenhauer D (eds) Süßwasserflora von Mitteleuropa, Band 2/1. Stuttgart/New York: Gustav Fischer Verlag.

Lamprinou V, Danielidis DB, Economou-Amilli A, Pantazidou A. 2012. Distribution survey of Cyanobacteria in three Greek caves of Peloponnesse. Inter J Speleol 41:267–272.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Komárek J. 2005. The biology of carbonate precipitation by cyanobacteria. In: Leadbeater BSC, Riding R. editors. Biomineralization in lower plants and animals. System Asso 30:73–90.

Mudimu O, Rybalka N, Bauersachs T, Friedl T, Schulz R. 2014. Influence of different CO2 concentrations on microalgae growth rate, α-tocopherol content and fatty acid composition. Geomicrobiol J 10:e100149.

Pentecost A. 2005. Travertine. Heidelberg: Springer.

Pentecost A, Riding R. 1986. Calcification of cyanobacteria. In: Leadbeater BSC, Riding R. editors. Biomineralization in lower plants and animals. System Asso 30:73–90.

Pentecost A, Bayari S, Yesertener C. 1997. Phototrophic microorganisms of the Pamukkale travertine, Turkey: Their distribution and influence on travertine deposition. Geomicrobiol J 14:269–283.

Pepe-Ranney C, Berelson WM, Corsetti F A, Treatis M, Spear JR. 2012. Cyanobacteria construction of hot spring siliceous stromatolites in Yellowstone National Park. Environ Microbiol 14:1182–1197.

Pfenzer WF, Wootke SL, Hall T. 1988. Microbial communities of Pyrenophora-infested wheat straw as examined by multivariate analysis. Microb Ecol 15:95–113.

Ploevie-Moraj A, Horvatinic N, Prime-Habdija B. 2002. Periphyton and its role inufa deposition in karstic waters (Plitvice Lakes, Croatia). Biologia 57:423–431.

Predkiwski F, Friedl T, Latala A. 2010. Identification of diatom isolates from the Gulf of Gdańsk: testing of species identifications using morphology, 18S rDNA sequencing and DNA barcodes of strains from the Culture Collection of Baltic Algae (CCBA). Oceanol Hydrobiol Studies, 39:3–20.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies, J, Glöckner, FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucl Acids Res 41:D590–D596.

Reichardt E. 1995. Die Kieselalgenflora (Bacillariophyceae) des Wachsenden Steins von Usterling. Berichte der Bayerischen Botanischen Gesellschaft 65:87–92.

Reichelt H. 1899. Über fossile Diatomeen aus Nordböhmern. Berichte der Naturforschenden Gesellschaft zu Leipzig 1899–1900:27–35.

Riding R. 1991. Classification of microbial carbonates. In: Riding R (ed) Calcareous Algae and Stromatolites. Berlin: Springer-Verlag, p21–51.

Romari K, Vaulot D. 2004. Composition and temporal variability of picoeukaryote communities at a coastal site of the English Channel from 18S rDNA sequences. Limnol Oceanogr 49: 784–798.

Rott E. 1994. Der Algenaufwuchs in der Oberen Alz (Oberbayern). Berichte der Naturwissenschaftlich-Medizinischen Vereins in Innbruck 81:229–253.

Sabater S. 1990. Composition and dynamics of a highly diverse diatom assemblage in a limestone stream. Hydrobiologia 190:43–53.

Schloss PD, Westcott SL, Rytuba T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541.

Schneider D, Reimer A, Hahlbrock A, Arp G, Reitner J, Daniel R. 2014. Metagenomic and metatranscriptomic analyses of bacterial communities derived from a calcifying karst water creek biofilm and tufa. Geomicrobiol J, submitted.

Sims PA, Mann DG, Medlin LK. 2006. Evolution of diatoms: insights from fossil, biological and molecular data. Phycologia 45:361–402.

Shiraishi F, Bissett A, De Beer D, Reimer A, Arp G. 2008a. Photosynthesis, Respiration and Exopolymer Calcium-Binding in Biofilm Calcification (Westerhöfer and Deinschwanger Creek, Germany). Geomicrobiol J 25:83–94.
Shiraishi F, Bissett A, De Beer D, Reimer A, Arp G. 2008b. Microbial effects on biofilm calcification, ambient water chemistry and stable isotope records (Westerhöfer Bach, Germany). Palaeogeogr Palaeoclimate Palaeoecol 262:91–106.

Sorhannus U. 2007. A nuclear-encoded small-subunit ribosomal RNA timescale for diatom evolution. Marine Micropaleontology 65:1–12.

Spitzer S, Brinkmann N, Reimer A, Ionescu D, Friedl T, de Beer D, Arp G. 2014. Effect of variable $\mu$CO$_2$ on Ca$^{2+}$ removal and potential calcification of cyanobacteria biofilms—An experimental microsensor study. Geomicrobiol J, accepted.

Stamatakis A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690.

Stock A, Breiner HW, Pachiadaki M, Edgecomb V, Filker S, La Cono V, Yakimov MM, Stoeck T. 2012. Microbial eukaryote life in the new hypersaline deep-sea basin Thetis. Extremophiles 16:21–34.

Stoeck T, Hayward B, Taylor GT 2006. A multiple PCR-primer approach to access the microeukaryotic diversity in environmental samples. Protist 157:31–43.

Talavera G, Castresana J. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. System Biol 56:564–577.

Tavera R, Novelo E, López S. 2013. Cyanoprokaryota (Cyanobacteria) in karst environments in Yucatán, México. Botan Sci 91:27–52.

Ter Braak CJF, Šmilauer P. 2002. CANOCO Reference Manual and CanoDraw for Windows User’s Guide: Software for Canonical Community Ordination (version 4.5). Ithaca, NY: Microcomputer Power.

Theriot EC, Cannone JJ, Gutell RR, Alverson AJ. 2009. The limits of nuclear-encoded SSU rDNA for resolving the diatom phylogeny. Euro J Phycol 44:277–290.

Thomazeau S, Houdan-Fourmont, A, Couté, A, Duval, C, Couloux, A, Rousseau, F, Bernard, C. 2010. The contribution of sub-Saharan african strains to the phylogeny of cyanobacteria: focusing on the Nostocaceae (Nostocales, Cyanobacteria). J Phycol 46:564–579.

Usdowski E, Hoefs J, Menschel G. 1979. Relationship between $^{13}$C and $^{18}$O fractionation and changes in major element composition in a recent calcite-depositing spring—a model of chemical variations with inorganic CaCO$_3$ precipitation. Earth Planet Sci Lett 42:267–276.

Wilmutte A. 1994. Molecular evolution and taxonomy of cyanobacteria. In: The molecular biology of cyanobacteria. In: Bryant DA, editor. The Netherlands: Kluwer Academic Publishers, p1–25.

Winsoborough BM, Golubič S. 2004. The role of diatoms in stromatolite growth: two examples from modern freshwater settings. J Phycol 23:195–201.

Zimmermann J, Jahn R, Gemeinholzer B. 2011. Barcoding diatoms: evaluation of the V4 subregion on the 18S rRNA gene, including new primers and protocols. Orga Diver Evol 11:173–192.

Zippel B, Neu TR. 2010. Characterization of glycoconjugates of extracellular polymeric substances in tufa-associated biofilms by using fluorescence lectin-binding analysis. Appl Environ Microbiol 77:505–516.