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# Assessing the diversity of pennate benthic diatoms in calcifying biofilms of hard water creeks

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## INTRODUCTION

Biofilms on calcareous tufa stromatolites in hardwater creeks are dominated by diatoms and cyanobacteria, producing high amounts of exopolymers that possibly play an important role in $CaCO_3$ nucleation and calcification processes (Arp et al. 2001, Pentecost & Bauld 1988, Zippel et al. 2006). Photosynthesis appears to have a significant impact on the carbonate equilibrium within the microenvironment of such biofilms (Shiraishi et al. 2006). However, the formation of calcareous tufa has otherwise been considered to be largely inorganic, i.e. physicochemically driven by $CO_2$ degassing rising $CaCO_3$ supersaturation (Herman & Lorah 1987, 1988). Tufa stromatolites are widespread in hardwater creeks. Here, karstification driven by soil-derived $CO_2$ leads to the formation of $Ca^{2+}$-rich, high-$pCO_2$-groundwaters which rapidly degas when the aquifer discharges to the subaerial environment.

In this ongoing project, the diversity of "key player" diatoms that are involved in stromatolite forming processes along a gradient of $pCO_2$ is assessed. Biofilm samples from the surfaces of tufa stromatolites (Fig. 1) were taken from two selected exemplary creeks in Germany, the Deinschwanger Bach (DB, Franconian Alb) and the Westerhöfer Bach (WB, Harz Mountains). WB is rich in $Mg^{2+}$ and $SO_4^{2-}$ compared to DB. Therefore, different diatom species compositions may be expected. The main focus of our study is to generate molecular signatures of diatoms from the calcareous biofilms and to establish a reference sequence database that enables rapid and unequivocal identification. This is essential to infer the spatial distribution of diatoms within the biofilm as well as seasonal variations in the diatom species composition. Culture-independent approaches as well as culture analyses were combined in order to assess the diatom diversity as completely as possible. Cultures of diatoms were also used for identification by morphology which was essential when no neighbouring sequences were available

In a second step of the project, Denaturing Gradient Gel Electrophoresis (DGGE) will be used to rapidly compare different creek sites and determine the seasonal variations in the diatom diversity of the biofilms. The spatial distribution of diatoms within the calcifying biofilms will be analysed by Fluorescence In Situ Hybridization (FISH). Cultures of diatoms will also be used for calcification experiments.

## MATERIAL & METHODS

The Deinschwanger Bach (DB) is located at the western rim of the Franconian Alb next to Nürnberg, Bavaria. Biofilm samples were taken in winter at eight different sampling sites. The Westerhöfer Bach (WB) is located in the westerly foreland of the Harz Mountains, Lower Saxony. Samples were taken in winter from five sites along

the first 325 metres of the creek. Biofilms (Fig. 1) were scratched from stones under sterile conditions. Biofilm material was used for establishing cultures which were maintained on diatom agarized medium (according to medium 11, SAG Göttingen, Germany) at 18°C under a light/dark regime of 14h:10 h at a photon fluency rate of about 25 µmol photons $m^{-2}$ $s^{-1}$ from white fluorescent bulbs. For DNA extraction, disruption of biofilm material and cell breakage was achieved by freeze-thaw cell lysis using liquid $N_2$ or bead beating, respectively (Minibeadbeater™, BioSpec, Bartlesville, USA,).18S rDNAs were amplified using PCR primers that selectively bind to diatom rRNA genes (primers sequences will be published elsewhere) and PCR products were purified using the NucleoSpin-Extract-Kit (Macherey-Nagel) and eluted in the buffer provided by the kit. In order to minimize PCR bias or the over representation of chimeric PCR products three independent PCR reactions were pooled and used for cloning. The purified PCR products were ligated into the TOPO® vector (TOPO TA Cloning Kit, Invitrogen) and cloned in competent *E. coli* cells. For the subsequent insert check white clones from Masterplates were directly used as template for amplification using the same PCR primer set as in the first PCR. Plasmid DNA of positive clones was extracted using the NucleoSpin-Plasmid-Kit (Macherey & Nagel). Sequence reactions were separated on a ABI 3100 capillary sequencer and the sequences assembled using the programme SeqAssem (Hepperle 2004). The 18S rDNA sequences were added to a reference database which contains over 8600 eukaryotic SSU rDNA sequences and is maintained in the ARB program (version 05.05.26, Ludwig et al. 2004, www.arb-home.de). Phylogenetic analyses were done on an alignment comprising only full length sequences which was extracted from the ARB program and used for calculating Maximum Likelihood trees using MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). Partial sequences were compared with publicly available sequences using the NCBI nucleotide-nucleotide blastn search tool (http://www.ncbi.nlm.nih.gov/BLAST/).

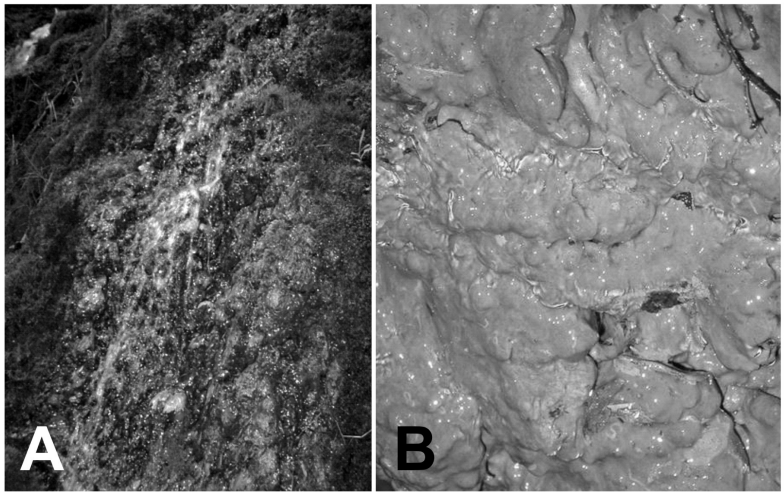


**Fig. 1. A.** Tufa stromatolites of hard water creeks investigated in this study (Westerhöfer Bach). **B.** Tufa covered by dense calcareous biofilm (Deinschwanger Bach).

## RESULTS

In the 18S rDNA phylogeny the diatoms from both hard water creeks were dispersed over 24 different lineages and clades within a large monophyletic clade representing the raphid pennate diatoms (Fig. 2A). In addition, three genera of araphid pennate diatoms, *Diatoma, Staurosira* and *Ulnaria*, were also found. Twenty of these lineages could be identified to the genus level by comparisons with already available sequences. High sequence similarities (≥ 98 %) of the creek diatoms with available sequences from the genera *Achnanthidium*, *Eolimna* and *Navicula* were found. However, for four lineages no next neighbouring sequences were available leaving them unidentified. The most dominant diatom detected by the culture approach was *Fistulifera pelliculosa* (Bréb. ex Kütz.) Lange-Bert. (99.5 % sequence similarity). In the culture-independent approach we considered a diatom lineage as significantly abundant, i.e. representing an important fraction of the biofilm biodiversity on the tufa stromatolites, when it was found at more than three sampling sites. Other strains that were found once, twice or three times only were regarded to contribute only randomly to the biofilm diversity.The significantly abundant lineages of diatoms were assigned to *Achnanthidium minutissimum* s.l., *Amphora*, *Gomphonema*-related, *Lyrella*-related, *Planothidium*, *Navicula* s.str., and a clade "unidentified 3". For the latter, no neighbouring sequences were available from public databases. A clear identification was further hampered because several genera appeared to be of multiple origins in the 18S rDNA phylogeny, e.g. *Nitzschia* (Fig. 2A). DB harboured a higher diversity of phylotypes than WB.

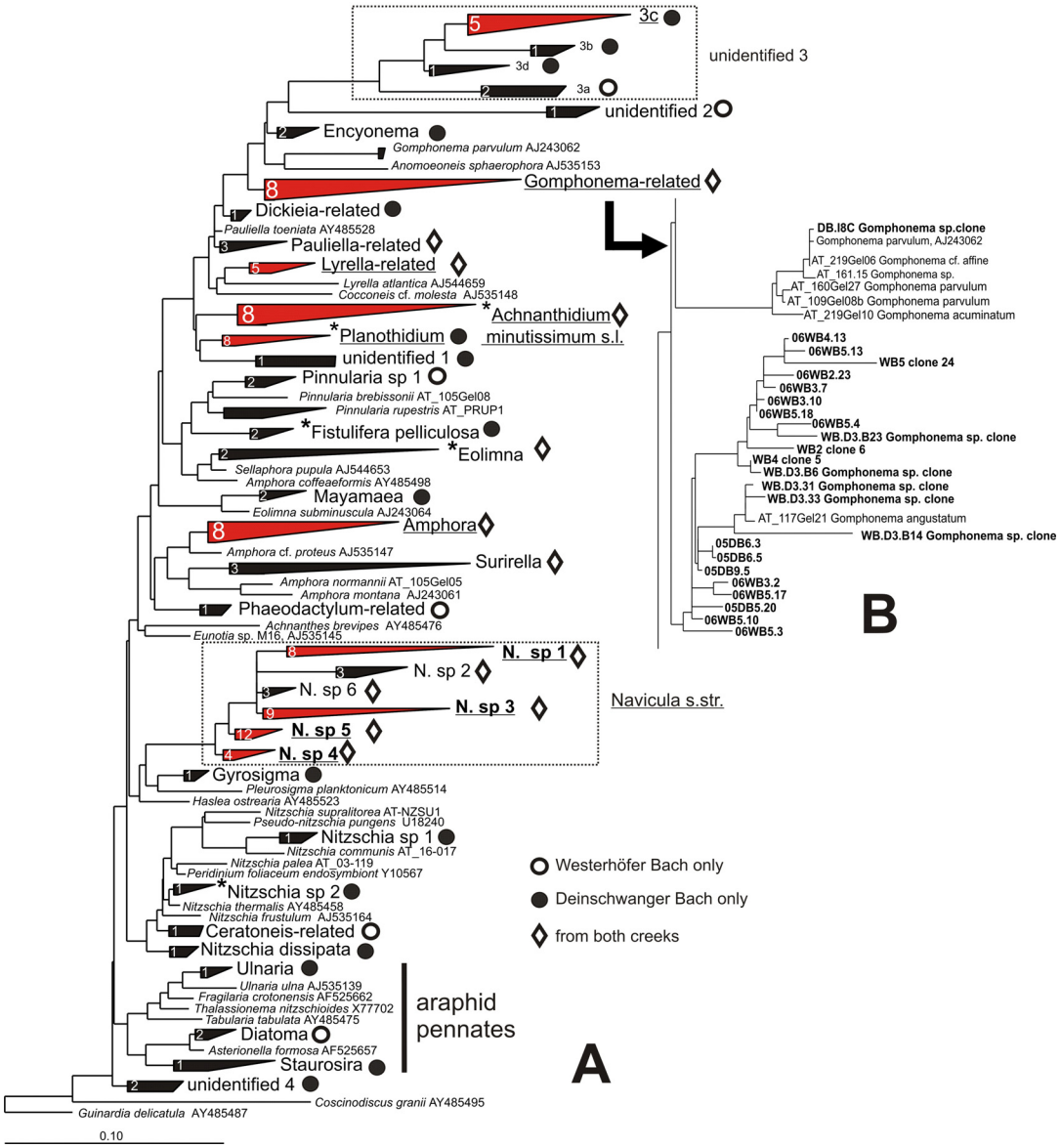


**Fig. 2. A.** 18S rDNA phylogeny of benthic pennate diatoms (Maximum likelihood, Bayesian analysis). Pennate diatoms detected in biofilms covering tufa stromatolites of two hardwater creeks, the Westerhöfer Bach and Deinschwanger Bach. Names of groups that were found more than three times are underlined. Groups of sequences were collapsed into triangles and the frequency, i.e. the number of sampling sites at which the members were found, is given. The presence of a diatom group at either both studied creeks or only one of both is indicated by symbols next to each clade. Next-neighbouring available reference sequences were from public databases (identified by their accession numbers) or from the AlgaTerra project (www.algaterra.org). For those genera that are marked by an asterisk cultures are available. **B.** Detail of the *Gomphonema* clade showing internal diversity and neighbouring sequences from GeneBank.

There were 14 additional lineages (although with low frequency, i.e. found at three or fewer sites) that were detected just in DB, but not in WB (Fig. 2A). Conversely, just six additional lineages were found to occur exclusively in WB (again with low frequency). Within most clades (representing genera) a considerable variation was found, e.g. in the *Gomphonema* clade (Fig. 2B). This may indicate that several species of the same genus have been present.

## DISCUSSION

Earlier identifications of diatoms from calcareous biofilms were based on morphological features of their frustules (Reichardt 1995, Rott 1994, Freytet & Verrecchia 1998) and resulted in clear names for species. In this study, it is also an important task to obtain

taxonomically correct species names for the identifications. For this the availability of diatom cultures will be indispensable in order examine their distinctive morphological features at the ultrastructural (SEM) level. Although we have failed to assign species or even sometimes genus names to the detected diatoms yet, our molecular approach has provided unique and unambiguous genomic signatures (phylotypes) of biofilm diatoms by which they can be easily distinguished and traced without time-consuming preparations or culturing. As soon as morphological features for the biofilm diatoms detected here will become available (e.g. cultures), it will be possible to test the phylogenetic significance of morphological characters used for species identification. Phylogenetic relationships within the raphid diatoms still need to be further clarified by molecular phylogenies. It appeared that of the large diversity of benthic pennate freshwater diatoms only a small fraction has been sequenced at the 18S rDNA level so far and this makes identifications by sequence comparisons difficult at present. We anticipate that our work may also initiate further studies to help in clarifying phylogenetic relationships of benthic pennate freshwater diatoms. At least for those diatoms which occur in calcifying biofilms our study has developed a reference data base that enables rapid unambiguous identifications for further studies.

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