Diversity of Microscopic Green Algae (Chlorophyta) in Calcifying Biofilms of Two Karstic Streams in Germany

LADISLAV HODAČ1,*, NICOLE BRINKMANN2, KATHRIN I. MOHR3, GERNOT ARP2, CHRISTINE HALLMANN1, JESSICA RAMM4, KAROLIN SPITZER1, and THOMAS FRIEDL1*

1Experimental Phycology and Culture Collection of Algae (SAG), Albrecht-von-Haller-Institute for Plant Sciences, Georg-August-University of Göttingen, Göttingen, Germany
2Geoscience Center, Georg-August-University of Göttingen, Göttingen, Germany
3Helmholtz Center for Infection Research, Braunschweig, Germany
4Department of Freshwater Conservation, Research Station Bad Saarow, Brandenburg University of Technology Cottbus-Senftenberg, Bad Saarow, Germany

Received August 2013; Accepted December 2013

For the first time the diversity of microscopic green algae (Chlorophyta) from calcified biofilms of karstic streams was analyzed using a combined approach based on pure cultures, i.e., 18S rRNA gene sequencing and microscopic analyses. Our study focused on two creeks in Germany. A considerable diversity of 34 species of green microalgae comprising three classes, the Trebouxiophyceae, Chlorophyceae and Ulvophyceae, was recovered. The biofilms of both streams were rather different in their species compositions which may take into account the exposure to different hydrochemical conditions. The shallow Westerhörfer creek harbored predominantly Trebouxiophyceae and exhibited higher Mg²⁺ and SO₄⁻² concentrations. In contrast, the deeper, longer and spatially more heterogeneous Deinschwanger creek harbored numerous species of Chlorophyceae. A lower number of species from the Ulvophyceae were spread on both studied streams. The closest relatives of the identified species were from other freshwater habitats, but mostly from phytoplankton. However, also several species we discovered from freshwater for the first time. The genus Gongrosira Küting, often reported from freshwater tufa-stromatolites, was found to represent most likely a collective morphotype formed by several genera nested within the Ulvophyceae.

**Keywords:** 18S rRNA phylogeny, Chlorophyceae, Chlorophyta, cultures, karst-water creeks, Trebouxiophyceae, Ulvophyceae

**Introduction**

Many karstic streams in Europe and elsewhere are characterized by calcium carbonate deposits which are biofilm-covered rock surfaces at the stream bed. These deposits are termed tufa stromatolites, defined as macroscopically laminated benthic microbial deposits produced by precipitation of minerals on organic tissue (Riding 1990). Biomineralization (biological processes) and inorganic precipitation may act together (Ford and Pedley 1996) or photosynthetic CO₂ assimilation by cyanobacteria, eukaryotic algae, and plants may be the primary cause for the carbonate precipitation (e.g., Hepperle and Krienitz 1996; Pia 1926, 1933; Wallner 1934).

Indeed, microsensor studies have demonstrated a photosynthetic control of CaCO₃ precipitation for biofilm-covered surfaces, while inorganically driven precipitation prevails e.g., at moss surfaces (Shiraishi et al. 2008a, 2008b). Microscopic studies revealed the dominance of filamentous cyanobacteria in the calcified biofilms of freshwater karst creeks (Brinkmann et al. this issue; Freytet and Plet 1996; Garcia-Pichel et al. 2004). But also diatoms, xanthophytes, red algae and other microscopic algae as well as bryophytes and microscopic fungi occur being associated with freshwater stromatolites (Bilan and Usov 2001; Brinkmann et al. this issue; Freytet and Verrecchia 1998; Heath et al. 1995; Winseborough and Golubić 1987).

In an ongoing larger study the possible roles of photosynthesis and respiration in calcification processes are being studied in detail at two exemplar karstic streams with prominent CO₂-degassing along their course, the Westerhörfer creek and
the Deinschwanger creek in Germany. Both tufa-forming streams attain high calcite supersaturation during their course downstream. The Westerhöfer creek, located in Middle Germany in the westerly Harz-foreland (51°45’N, 10°5’E), is 325 m long and less than 2 m wide and has its source in limestones and evaporites of the Middle Triassic Muschelkalk Group. The Deinschwanger creek is located in southern Germany at the western rim of the Franconian Alb (49°23’N, 11°28’E). It is fed by three main springs and a number of side springs, most of them discharging from the Upper Jurassic Weißjura-Group aquifer. Compared to the Deinschwanger creek the Westerhöfer creek is rich in Mg\(^{2+}\) and SO\(_4^{2-}\).

Microscopy of biofilm samples from both creeks revealed cyanobacteria and diatoms as the dominant algae (Brinkmann et al. this issue), but other micro-algae were found only rarely or not at all. Concurrently with a study on the biodiversity of the cyanobacteria and diatoms from both creeks (Brinkmann et al. this issue), cultures of green algae were developed. Interestingly, in the enrichment cultures an unexpected variety of green micro-algae appeared besides numerous cyanobacteria and diatoms. It is not known yet whether there are green algal taxa with strict or even any preference for calcifying biofilms. Their presence in calcifying biofilms may even be entirely given by accident. There is an expectation that the most green algae in calcifying biofilms could originate from soils and other aerial and subaerial habitats. Here we report about the phylogenetic and morphological diversity of these green microalgae.

Materials and Methods

**Sampling, Culturing, and Microscopy**

Biofilms from both, the Westerhöfer and Deinschwanger creeks, were collected in the spring or early summer (May/June) in 2005–2007. Five sites of the Westerhöfer creek (abbreviated as WB) and eight sites from Deinschwanger creek (abbreviated as DB) were selected for sampling of apparently algae-dominated biofilms (Table 1). For starting enrichment cultures all samples from the WB were pooled together because it is rather short and only a short segment (about 350 m) of the creek was investigated, whereas for the DB the samples from different locations were analyzed separately to better reflect its higher habitat heterogeneity.

The biofilms were scratched off from stone surfaces using an ethanol-sterilized knife or spatula and transferred to 1.5 ml reaction tubes which were cooled until further processing in the laboratory the following day. A spatula-full of biofilm material was then transferred into different standard liquid growth media. Because our initial main focus was to establish cultures of cyanobacteria and diatoms, growth media provoking the development of these algae were used, i.e., BG11, BG11 without citrate, Z, Z 45/4, and ES (http://www.uni-goettingen.de/de/186449.html). Apart from the expected growth of cyanobacteria and diatoms, also intensive green algal growth was observed after about 5–10 days of cultivation.

Putative green algal colonies were transferred on 1.5% agar plates with 3N BBM+V medium (http://www.uni-goettingen.de/de/186449.html). After incubation for another 4–8 weeks, all green algal morphotypes were isolated into unialgal cultures by several rounds of streaking on fresh agar plates of 3N BBM+V medium using sterile platinum needles. Finally, the unialgal isolates were microscopically checked for purity and further maintained on agar slants at 18°C under a light/dark regime of 14:10-h and a photon flow rate of about 25 μmol photons m\(^{-2}\) s\(^{-1}\) from white fluorescent bulbs.

A total of 77 pure cultures was established, out of which 45 were studied in more detail (Supplementary Table 1). Nineteen of them were made publicly available, i.e., accessioned by the SAG culture collection (Göttingen University, Germany; www.epscs.uni-goettingen.de). For microscopy an Olympus BX60 microscope (Tokyo, Japan) with Nomarski DIC optics and an attached ColorView III camera (Soft Imaging System, Münster, Germany) was used. Micrographs were processed using Cell^D image software (Soft Imaging System, Münster, Germany).

**DNA Extraction, PCR, and Sequencing**

DNA was extracted from all unialgal isolates (Supplementary Table 1) using the Invisorb Spin Plant Kit (Stratec, Berlin, Germany) as recommended by the manufacturer. Nuclear-encoded 18S rRNA genes were amplified using primers NS1 and 18L (Hamby et al. 1988). For some strains the 18S and ITS1-5.8S-ITS2 rRNA gene region was amplified using primers NS1 and LR1850 (Friedl 1996). If a culture was suspected to be contaminated by fungi or bacteria, PCR primer 1650R (5’-TCACCACGACAAYCAT-5’; pos. 1652-1636 of the 18S rRNA gene sequence of Chlorella vulgaris SAG 211-11b, FM205832) which preferentially binds to members of Chlorophyta, was used as the reverse PCR primer.

Conditions for PCR and cycle sequencing reactions and the standard set of sequencing primers were as described earlier (Mikhailyuk et al. 2008). The newly determined sequences were deposited in GenBank under the accession numbers KF144164 - KF144240 (Supplementary Table 1). In addition also the 18S rRNA gene sequences for the following strains were determined as references: Chlorococcum vacuolatum Starr SAG 213-8 (acc. no. KF144189), Dilahyttium printzii (Visher) Bourellly SAG 467-1 (acc. no. KF144198), Scotothrix phaeaea giberosa (Vodenicarv & Benderliev) Wujek & R.H. Thompson SAG 75.80 (acc. no. KF144239) and S. lemnae (Punčehárová) Wujek & R.H.Thompson SAG 240-1 (acc. no. KF144230).

**Phylogenetic Analyses**

To search for the closest neighboring relatives of our isolates their sequences were compared to those from reference strains at NCBI (http://www.ncbi.nlm.nih.gov) using BLASTn queries (Altschul et al. 1997). Only almost full neighboring 18S rRNA gene sequences were downloaded together with a selection of reference sequences to better represent the green algal classes Trebouxioiphyceae, Chlorophyceae and Ulvophyceae as well as additional
green algal lineages and aligned using MAFFT, ver. 6 (Katoh and Toh 2008) available online at http://mafft.cbrc.jp/alignment/server/index.html. Three sequence data sets were constructed after the alignments were manually refined using BioEdit (Hall 1999).

The sequence data set of Trebouxiophyceae (Figure 1) contained 162 sequences and was 1766 positions long with 781/562 variable/parsimony informative sites, that of Chlorophyceae (Figures 2 and 3) contained 238 sequences and was 1797 positions long with 938/653 variable/parsimony informative sites, and that of Ulvophyceae (Figure 4) 88 sequences and was 1785 positions long with 953/813 variable/parsimony informative sites. The GTR+Γ+I model was selected as the best fitting model of nucleotide substitution for all three sequence data sets as based on the AIC criterion using jModelTest 0.1.1 (Posada 2008). Phylogenetic trees were calculated using maximum likelihood with the program RAxML 7.0.4 (Stamatakis et al. 2008) and Bayesian phylogenetic inference with MrBayes 3.2.1 x64 (Ronquist et al. 2012). For the latter, two MCMC runs for three million generations each were employed with one cold and three heated chains with trees sampled every 100 generations.

Confidence values for the obtained groups (internal edge support) were inferred from the rapid bootstrapping algorithm (100 replicates) as implemented in RAxML and from Bayesian posterior probabilities using MrBayes 3.2.1 x64 (Ronquist et al. 2012). Pairwise sequence similarities from p-distances were determined as an additional measure of the relatedness of our isolates to certain reference strains/sequences under the Kimura 2-parameter model.

Table 1. Distribution of the recovered green algal species at the sampling sites

| Class          | Species                  | DB1 | DB2 | DB3 | DB4 | DB5 | DB6 | DB9 | DBS | WB  |
|----------------|--------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Trebouxiophyceae | *Chlorella* sp.          | 3   | 3   | 2   | 1   | 11  | 2   | 4   | 19  |
|                | *Coccomyxa* cf. *pringsheimii* |     |     |     |     |     |     |     |     |
|                | *Coccomyxa* cf. *simplex* |     |     |     |     |     |     |     |     |
|                | *Elliptochloris subphaerica* |     |     |     |     |     |     |     |     |
|                | *Marvania* sp.           |     |     |     |     |     |     |     |     |
|                | *Muriella terestris***   |     |     |     |     |     |     |     |     |
|                | *Neocystis* cf. *mucosa* |     |     |     |     |     |     |     |     |
|                | *Stichococcus* *bacillaris*** |     |     |     |     |     |     |     |     |
|                | *Stichococcus* cf. *deasonii* |     |     |     |     |     |     |     |     |
|                | *Stichococcus* *mirabilis*** |     |     |     |     |     |     |     |     |
|                | *Stichococcus* sp.1***   |     |     |     |     |     |     |     |     |
|                | *Stichococcus* sp.2     |     |     |     |     |     |     |     |     |
|                | *Stichococcus* sp.3     |     |     |     |     |     |     |     |     |
|                | *Stichococcus* sp.4     |     |     |     |     |     |     |     |     |
| Chlorophyceae  | *Acutodesmus* *obliquus*** |     |     |     |     |     |     |     |     |
|                | *Bracteacoccus* *aerius*-relative |     |     |     |     |     |     |     |     |
|                | *Bracteacoccus* sp.      |     |     |     |     |     |     |     |     |
|                | *Chlamydomonas* sp.      |     |     |     |     |     |     |     |     |
|                | *Chlamydomodium* sp.     |     |     |     |     |     |     |     |     |
|                | *Chlorococcum* *minutum*-relative |     |     |     |     |     |     |     |     |
|                | *Chlorococcum* *sphacocum* |     |     |     |     |     |     |     |     |
|                | *Chlorococcum* *ellipsoidalum*-relative1 |     |     |     |     |     |     |     |     |
|                | *Chlorococcum* *ellipsoidalum*-relative2 |     |     |     |     |     |     |     |     |
|                | *Desmodesmus* cf. *armatus* |     |     |     |     |     |     |     |     |
|                | *Monoraphidium* cf. *dybowskii* |     |     |     |     |     |     |     |     |
|                | *Mychonastes* cf. *homochaera* |     |     |     |     |     |     |     |     |
|                | *Mychonastes* sp.*      |     |     |     |     |     |     |     |     |
|                | *Pseudomuriella* cf. *schumacherensis* |     |     |     |     |     |     |     |     |
|                | *Scenedesmaceae* sp.     |     |     |     |     |     |     |     |     |
| Ulvophyceae    | *Desmochloris* cf. *halophila* |     |     |     |     |     |     |     |     |
|                | *Dilabifilum* *prinzii*** |     |     |     |     |     |     |     |     |
|                | *Hazenia* *mirabilis* |     |     |     |     |     |     |     |     |
|                | *Pseudodocloniopsis* *botryoides* |     |     |     |     |     |     |     |     |
|                | *Pseudodoclonium* *akinetum* |     |     |     |     |     |     |     |     |

Note: The recovered species were distributed at eight sampling sites of the Deinschwanger creek (DB) and the pooled sample from the Westerhöfer creek (WB, from five sites). Numbers below sampling sites are the corresponding values [log IAP/KT] of calcite saturation index (SICalcite, for explanation see Arp et al. 2010) and the total number of species recovered per site. Two asterisks next to a species name indicate 100%, a single asterisk 99.9% sequence identity with a reference strain.
Fig. 1. Maximum-likelihood (ML) phylogeny of 18S rRNA gene sequences of green algal species from the class Trebouxiophyceae isolated from biofilms of two freshwater creeks (highlighted sequence names) and other members of the Trebouxiophyceae as references. Additional members of Oocystaceae (Trebouxiophyceae) as well as other classes of the Chlorophyta, the Chlorophyceae, Ulvophyceae and Chlorodendrophycaceae (outgroup), are included for comparisons and to root the tree (for their accession numbers see Supplementary Table 4). Additional outgroups including Picocystis salinarum and Nephroselmidophyceae were removed from the figure due to lack of space (all outgroups were the same as in Figure 2, for accession numbers see Supplementary Table 4). Numbers at internal branches indicate support from bootstrap tests using ML (left) and posterior probabilities from Bayesian analysis (BI; right). Thick lines mark internal branches that were resolved by both, the ML and the BI tree topologies. Grey boxes highlight the clades comprising the new isolates.

Hodač et al.
Fig. 2. Maximum-likelihood (ML) phylogeny of 18S rRNA gene sequences of green algal species from the class Chlorophyceae, Chlamydomonadales clade, isolated from biofilms of two freshwater creeks (highlighted sequence names) and other members of the Chlorophyceae, Chlamydomonadales clade, as references. Additional members of Chlorophyceae as well as other classes of the Chlorophyta, the Trebouxiophyceae, Ulvophyceae, Chlorodendrophyceae; *Picocystis salinarum* and Nephroselmidophyceae (out-group), are included for comparisons and to root the tree (for their accession numbers see Supplementary Table 4). Numbers at internal branches indicate support from bootstrap tests using ML (left) and posterior probabilities from Bayesian analysis (BI; right). Thick lines mark internal branches that were resolved by both, the ML and the BI tree topologies. Grey boxes highlight the clades comprising the new isolates.
Fig. 3. Maximum-likelihood (ML) phylogeny of 18S rRNA gene sequences of green algal species from the class Chlorophyceae, Sphaeropleales clade, isolated from biofilms of two freshwater creeks (highlighted sequence names) and other members of the Chlorophyceae, Sphaeropleales clade, as references. Additional members of Chlorophyceae as well as other classes of the Chlorophyta, the Trebouxiophyceae, Ulvophyceae, Chlorodendrophyceae; *Picocystis salinarum* and Nephroselmidophyceae (outgroup), are included for comparisons and to root the tree (for their accession numbers see Supplementary Table 4). Numbers at internal branches indicate support from bootstrap tests using ML (left) and posterior probabilities from Bayesian analysis (BI; right). Thick lines mark internal branches that were resolved by both, the ML and the BI tree topologies. Grey boxes highlight the clades comprising the new isolates.
model with the programme MEGA5 (Tamura et al. 2011).

Fig. 4. Maximum-likelihood (ML) phylogeny of 18S rRNA gene sequences of green algal species from the class Ulvophyceae, isolated from biofilms of two freshwater creeks (highlighted sequence names) and other members of the Ulvophyceae as references. Additional members of other classes and lineages of the Chlorophyta are included for comparisons. The phylogeny was rooted with two members of the Nephroselmidophyceae. Numbers at internal branches indicate support from bootstrap tests using ML (left) and posterior probabilities from Bayesian analysis (BI; right). Thick lines mark internal branches that were resolved by both, the ML and the BI tree topologies. Grey boxes highlight the clades comprising the new isolates.

Statistical Analysis

The distribution of all major clades of green algae at studied sampling sites was investigated using multivariate ordination by Nonmetric Multidimensional Scaling (NMDS); the analysis was conducted in PAST 2.06 (Hammer et al. 2011). The input data corresponded to a matrix similar as in Tables 1 and 2 summarizing the presence/absence of isolates at particular sampling sites as identified by 18S rRNA gene sequence comparisons.

Results

A total of 77 isolates have been obtained from both creeks (Supplementary Table 1). Phylogenetic analyses of 18S
rRNA gene sequences determined for each isolate distinguished 34 distinct lineages supposedly corresponding to species. They were distributed on three classes of green algae, the Trebouxiophyceae (14 species; Figure 1), the Chlorophyceae (15 species; Figures 2, 3) and the Ulvophyceae (5 species; Figure 4). Forty-five isolates have been selected for phylogenetic and microscopic analyses in this article (Figures 1–7; Supplementary Tables 1, 2). The other isolates shared identical (partial) sequences and, therefore, were suspected to represent the same species as the selected 45 isolates. Morphological features of the 34 species (77 isolates) were investigated and are summarized in Supplementary Table 2.

### Diversity of Trebouxiophyceae

The recovered Trebouxiophyceae isolates were distributed on four major clades of the class (Figure 1). Those isolates with a *Stichococcus*-like rod shaped morphology (Figures 5a and 5b) were distributed in seven distinct lineages within the Prasiola-clade. Based on high sequence similarities (99.9 or 100% as determined from p-distances) and short genetic distances with named reference sequences in the phylogenetic analyses (Figure 1) two closely related isolates, WB13 and WB74 (Figure 5a), were identified as *S. bacillaris* and isolate WB69 as *S. mirabilis* (Table 1; Supplementary Table 3).

For the other *Stichococcus*-like isolates sequence similarities with named references were lower or no closer named references were available at all. Isolate WB38 was named *S. cf. deasonii* as its next named reference was *S. deasonii*. For six isolates which were distributed on four independent lineages of the Prasiola clade (Figure 1) no close named relatives were available and, therefore, they were named *Stichococcus* sp. 1 (isolates DB6-27, WB65, and SAG 2407), *S. sp. 2* (isolate D4-2A), *S. sp. 3* (isolate SAG 2408) and *S. sp. 4* (isolate SAG 2406; Figure 5b). Based on morphology all seven lineages may be assigned to *Stichococcus*, but in the 18S rRNA gene phylogenies the genus was paraphyletic with many other coccoid and filamentous members of the Prasiola clade (Figure 1). The recovered seven lineages of *Stichococcus*-like Trebouxiophyceae may correspond to seven distinct species, but their assignment to a single genus *Stichococcus* is not adequate. The genus as currently circumscribed may in fact encompass several distinct genera.

The unicellular spherical trebouxiophytes (Figures 5c-5f) represented three distinct lineages within the Chlorellales clade (Figure 1). The two isolates RK52 (Figure 5c) and D11-2 (Figure 5d) exhibited relatively large rounded cells with cup-shaped chloroplast and a single pyrenoid with starch grains attached to it, i.e., the *Chlorella* morphotype (Supplementary Table 2). Both isolates had sequences highly similar to each other and their closest neighbors (99.9% sequence similarity) were several unidentified *Chlorella* sp. strains. Both isolates represented the most frequently recovered green alga in our study, i.e., there was a total of 12 strains with almost identical 18S rRNA sequences and identical morphology representing the species (Supplementary Tables 1 and 2).

Resolution within the clade corresponding to the Chlorellaceae in the 18S rRNA phylogeny was low (Figure 1). Both *Chlorella*-like isolates were distinct from the authentic (type) strain of the genus, *C. vulgaris* SAG 211-11b, by a relatively large genetic distance (Figure 1), but a closer assignment to any genus of Chlorellaceae was impossible. *Hindakia* was the closest relative (Figure 1), but with a relatively low sequence similarity of 99.7%.

Two other trebouxiophyte species with spherical cells differed from the *Chlorella* morphotype by their smaller cells with a single band-shaped chloroplast without pyrenoid (Supplementary Table 2; Figures 5e, 5f). They were distributed in two distinct lineages outside of Chlorellaceae, but within the clade representing the Chlorella (Figure 1). Two isolates (D6-DB2 and SAG 2390) were assigned to *Muriella terrestris* (Figure 5e) due to low p-distances/high sequence similarities (99.9 and 100%) with strain ASIB V38 (acc. no. AB012845) which has been isolated from soil (Gärtner 2004).

Both our isolates also had the same high sequence similarity with an unidentified strain of *Muriella* from freshwater phytoplankton (AS2-4, acc. no. AY195969; Fawley et al. 2004). Therefore, we assume that both our isolates as well as
Strains ASIB V38 and AS2-4 represent the same species, *M. terrestris*. The other species, represented by isolate WB67 (Figure 5f), shared high sequence similarities with unidentified *Nannochloris*-like strains which together shared a well-supported monophyletic origin with *Marvania geminata*. Because of the relatively low sequence similarity (97.8%) with the reference sequence of *M. geminata* our isolate probably represents another yet still undescribed species of *Marvania*.

Other isolated trebouxiophytes exhibited reniform (*Neocystis*-like; Figure 5g), elliptic to nearly spherical (*Elliptochloris*-like; Figure 5h) or elongated to elliptic (*Coccomyxa*-like; Figure 5i) cell shapes. The next relative to isolate SAG 2405, characterized by its mucilaginous colonies and reniform cell shape (Figure 5g), was *Neocystis mucosa* strain KR 1989/14 (acc. no. HM565928), therefore it was termed *Neocystis* cf. *mucosa*. Another *Neocystis* strain, *Neocystis brevis* CAUP D802 (acc. no. HQ287929), was slightly more distant to SAG 2405 and the whole clade of *Neocystis*-like trebouxiophytes was highly supported. Isolate WB5-D1e (Figure 5h) was identified as *Elliptochloris subsphaerica* based...
on its high sequence similarity of 99.9% with *E. subsphaerica* strain SAG 2202 (acc. no. FJ648518).

The same short p-distance/high sequence similarity was with unidentified *Elliptochloris* strain SAG 2117 (acc. no. FJ648515). The whole clade comprising the three *Elliptochloris* sequences was well supported (Figure 1). Two more species exhibited a typical *Coccomyxa*-like morphology (Figure 5i) and were nested within a well-supported clade consisting of several *Coccomyxa* strains. The one species, represented by the single isolate WB40, was most closely related to strain *Pseudococcomyxa simplex* UTEX 274 (acc. no. FJ648514). The other species was represented by four isolates, retrieved from both creeks (Supplementary Table 1). Out of them for isolate WB28 an almost full 18S rRNA gene sequence was determined and strain *Coccomyxa pringsheimii* SAG 69.80 (acc. no. AY762603) represented its closest neighboring sequence.

**Fig. 6.** Morphology of green algal isolates of the class Chlorophyceae. Chlamydomonadales (a)–(f); Sphaeropleales (g)–(h). *Chlorococcum sphacorum* SAG 2398 (a), *Chlorococcum minutum*-relative SAG 2399 (b), *Chlorococcum ellipsoideum*-relative1 GRK6-DB5 (c), *Chlorococcum ellipsoideum*-relative2 SAG 2400 (d), *Chlorococcum ellipsoideum*-relative2 GRK6-DB6, zoospores (e), *Chlamydomonas* sp. isolate RK68 (f), *Desmodesmus* cf. *armatus* isolate RK43 (g), *Acutodesmus obliquus* isolate D22-6-2B (h), *Scenedesmaceae* sp. isolate RK49 (i). Scale bars, 10 μm.
Diversity of Chlorophyceae

Isolates assigned to the class Chlorophyceae were distributed in two clades representing the orders Chlamydomonadales and Sphaeropleales (Figure 2 and Figure 3). The isolated members of Chlamydomonadales exhibited two morphological types, i.e., *Chlorococcum*-like large spherical cells with a single large chloroplast (Figure 6a–6e), *Chlamydomonas*-like monadoid biflagellated cells (Figure 6f), those of Sphaeropleales four morphological types, i.e., *Scenedesmus*-like elongated cells with acute ends (Figures 6h–6i), *Bracteacoccus*-like spherical cells with numerous discoidal parietal chloroplasts without a pyrenoid (Figure 7a, 7b), *Mychonastes* or *Nannochloris* like small spherical cells with a single chloroplast without a pyrenoid (Figures 7c, 7d), and *Monoraphidiun*-like fusiform cells.

**Fig. 7.** Morphology of green algal isolates of the classes Chlorophyceae and Ulvophyceae. Chlorophyceae, Sphaeropleales (a)–(d); Ulvophyceae (e)–(i). *Pseudomuriella cf. schumacherensis* RK3 (a), *Bracteacoccus* sp. DB9-3 (b), *Mychonastes* cf. homosphaera RK48 (c), *Mychonastes* sp. isolate DB6-29 (d), *Hazenia mirabilis* strain SAG 2396 (e), *Pseudendocloniopsis botryoides* SAG 2394, coccoid stage (f), *Pseudendocloniopsis botryoides* DB6-19, filamentous stage (g), *Pseudendoclonium akinetum* SAG 2404 (h), *Dilabifilum printzii* isolate WB41 (i). Scale bars, 10 µm.

**Diversity of Chlorophyceae**

Isolates assigned to the class Chlorophyceae were distributed in two clades representing the orders Chlamydomonadales and Sphaeropleales (Figure 2 and Figure 3). The isolated members of Chlamydomonadales exhibited two morphological types, i.e., *Chlorococcum*-like large spherical cells with a single large chloroplast (Figure 6a–6e), *Chlamydomonas*-like monadoid biflagellated cells (Figure 6f), those of Sphaeropleales four morphological types, i.e., *Scenedesmus*-like elongated cells with acute ends (Figures 6h–6i), *Bracteacoccus*-like spherical cells with numerous discoidal parietal chloroplasts without a pyrenoid (Figure 7a, 7b), *Mychonastes* or *Nannochloris* like small spherical cells with a single chloroplast without a pyrenoid (Figures 7c, 7d), and *Monoraphidiun*-like fusiform cells.
**Chlamydomonadales**

The *Chlorococcum*-like isolates were distributed in four distinct clades within the larger and highly supported *Stephanosphaerina*-clade in 18S rRNA gene phylogenies (Figure 2). One clade was formed by isolate RK50 and strain SAG 2402 and their named relative *Chlamydomonas vacuolatum* (acc. no. M63001) and, therefore, both were assigned to the genus *Chlamydomonas*. Another sequence, but from an unidentified strain, *Chlamydomonadales* sp. KMMCC:EC-34, was even closer with the former two strains (Figure 2). A second well supported clade included the isolates GRK6-DB5, GRK6-DB6, SAG 2400 and SAG 2401. Except for the latter two, which had almost identical sequences, they were all distant to each other and had sequences from unidentified strains as their next closest relatives. The next named closest relatives to *Chlorococcus ellipsoideum* (acc. no. U70586) and, therefore, we named our isolates species distinct from them and, therefore, we assigned isolate SAG 2398 to *C. sphacosum* (Figure 6a; Table 1, Supplementary Table 2). Both strains, SAG 2398 and SAG 66.80 (acc. no. JN968580), also shared 99.9% sequence similarity to each other and had sequences from unidentified species of the clade. A third clade contained a single isolate, SAG 2399, which was within a well-supported clade together with several named closest relatives (Figure 2). Except for *Chlorococcum sphacosum* SAG 66.80 (which corresponds to the authentic strain of the type of the species), our isolate was morphologically rather distinct from them and, therefore, we assigned isolate SAG 2398 to *C. sphacosum* (Figure 6a; Table 1, Supplementary Table 2). Both strains, SAG 2398 and SAG 66.80 (acc. no. JN968580), also shared 99.9% sequence similarity (Table 1, Supplementary Table 3).

Finally, strain SAG 2399 (Figure 6b) was nested within another distinct and highly supported clade with *C. vacuolatum* SAG 213-8 (acc. no. KF144189) and *C. minutum* ASIB T50 (acc. no. JN968585) as the closest named relatives (Figure 2). Because it appeared more similar in its morphology to *C. minutum* (Supplementary Table 2) we named strain SAG 2399 *Chlorococcum minutum*-relative. The *Chlamydomonas*-like monadoid isolates RK68 (Figure 6f) and DB6-shared identical (partial) sequences and isolate RK68 was within the well-supported *Reinhardtinia*-clade in the phylogenetic analyses (Figure 2). Its closest named relatives were three species of *Chlamydomonas* and *Volvox carteri*. Because it exhibited solitary cells with a typical *Chlamydomonas* morphology we assigned it to that genus, but could not identify it to species level.

**Sphaeropleales**

The *Scenedesmus*-like isolates were distributed in three independent lineages (species) of the *Scenedesmaceae* clade (Sphaeropleales; Figure 3). *Desmodesmus* cf. *armatus* was with six isolates among the most frequently recovered green algal species of our study (Supplementary Table 1). Both isolates, RK43 (Figure 6g) and strain SAG 2395, for which almost full 18S rRNA gene sequences were determined, formed a highly supported clade together with named reference strain, *Desmodesmus armatus* CCAP 276/4A (acc. no. FR865727) and, therefore, were assigned the isolates to this species (Figure 3). Isolate D22-6-2B (Figure 6h) shared an identical sequence with reference strain *Acutodesmus obliquus* CCAP 276/49 (acc. no. FR865726) and was assigned to this species.

Finally, isolate RK49 (Figure 6i) could not be assigned to a certain genus of the *Scenedesmaceae* clade because it was distinct from all named reference strains of that clade, albeit there was weak support for a closer relationship with species of *Acutodesmus* (Figure 3). The three *Bracteacoccus*-like isolates were distributed in two independent clades of the *Sphaeropleales* representing the genera *Bracteacoccus* and *Pseudomuriella* (Figure 3). The next closest named reference for isolate RK3 (Figure 7a) was *Pseudomuriella schumacherensis* SAG 2137 (acc. no. HQ29768) but with a sequence similarity < 99.9% and, therefore, we named our isolate P. cf. *schumacherensis*.

Despite their morphological similarities with the *P. cf. schumacherensis* isolate the two other *Bracteacoccus*-like isolates, SAG 2403 and DB9-3 (Figure 7b), were in a distinct clade of the 18S rRNA gene phylogeny, i.e. the *Bracteococcaceae* clade. Strain SAG 2403 had a close named relative, *Bracteococcaceae aerius* UTEX 1250 (Figure 3), but shared less than 99.9% sequence similarity from p-distances with the latter and, thus, was named *Bracteacoccus cf. aerius*. In contrast, isolate DB9-3 had no named closest relative; it was most closely to an unidentified *Bracteococcus* strain (KF-2011f), but rather distant from *B. cf. aerius* (Figure 3). Both *Mychonastes/Nannochloris*-like isolates, RK48 and DB6-29 (Figures 7c and 7d), were within a single well supported clade, representing the genus *Mychonastes* within the Chlorophyceae (Figure 3).

Isolate RK48 had *Mychonastes homosphaera* CAUP H6501 (which represents the authentic strain of the species) as its closest named relative, but with a sequence similarity of less than 99.9% (Supplementary Table 3) and, therefore, was named *Mychonastes cf. homosphaera*. *Mychonastes*-like isolate DB6-29 was distant from RK48 and had an unidentified *Mychonastes* strain, Itas 9/21 14-8w (acc. no. AY543066) as closest relative (with 99.9% sequence similarity) and, therefore, was named *Mychonastes* sp. (Figure 3).

Several species of *Mychonastes* have recently been described as common members of freshwater phytoplankton (Krienitz et al. 2011), but our isolates were more distant to those and, therefore, were not included in our phylogenetic analyses. The *Monoraphidium*-like creek biofilm isolate, strain SAG 2393, was most closely related (with sequence similarity <99.9%) to two reference strains named *M. dybowskii* within the *Selenastraceae* clade and, therefore, was named *M. cf. dybowskii*.

**Diversity of Ulvophyceae**

Five species from creek biofilms were members of the Ulvophyceae. Interestingly, out of them four were of pseudo-filamentous organization, i.e., formed only short filaments and also coccoid stages, whereas only one exhibited a purely coccoid vegetative stage. This was in contrast to our isolates of Trebouxiophyceae and Chlorophyceae which were all of coccoid or monadoid organization. The pseudo-filamentous isolates exhibited characteristic cup-shaped chloroplasts with one or two prominent pyrenoids (Figures 7h, 7i).
filamentous species were members of the order Ulotrichales, one belonged together with the coccoid isolate to the Ulvales (Figure 4). Filamentous strain SAG 2394 (Figure 7e) had a rather small genetic distance to Hazenia mirabilis UTEX LB 846 (acc. no. AF387156) and, therefore, was assigned to this species (Figure 4). Pseudendoclonium basiliense UTEX 2593 and two species of Gloeotilopsis formed up a clade with the former two strains which, however, received only insignificant support values (Figure 4).

Another pseudo-filamentous species we recovered was Pseudendocloniopsis botryoides; it was with nine isolates (with identical partial sequences and morphology) the second most frequently retrieved green algal species (Supplementary Table 1). As represented by isolate SAG 2394 the close relationship with two available strains of Pseudendocloniopsis botryoides was well supported and, therefore, SAG 2394 was assigned to this species (Figure 4). SAG 2394 may also form coccoid stages (Figure 7f), a feature shared with Planophila laetevirens SAG 2008, which also was its next closest relative in the 18S rRNA gene phylogeny (Figure 4). Morphology of P. botryoides was studied at isolate DB6-19 in more detail (Figure 7g; Supplementary Table 2).

The filamentous isolate SAG 2404 (Figure 7h) was a very closely relative to Pseudendoclonium akinetum UTEX 1912 and also two species of Trichosarcina were their next closest relatives, but these relationships did not receive significant support values (Figure 4). Finally, for another pseudo-filamentous species, Dilabilifilum printzii, a total of seven isolates was established (Supplementary Table 1); it was the third most frequently recovered green algal species in our study. Unfortunately, we failed to obtain an almost full 18S rRNA gene sequence for these isolates. The partial sequence of isolate WB41 shared 99.9% similarity with the sequence (acc. no. KF144198) of D. printzii strain SAG 467-1 and, therefore, we assigned our isolate to this species.

Morphological features of isolate WB41 are presented in Figure 7f and Supplementary Table 2. D. printzii SAG 467-1 had an independent position within the clade representing the Ulvales in our 18S rRNA gene phylogeny (Figure 4). The single coccoid Ulvophyceae isolate SAG 2397 clearly fell within a subclade of the Ulvales comprising the coccoid genera Chlorocystis, Desmochloris and Halochlorococcus (Figure 4). Strain SAG 2397 had short genetic distances with two strains of Desmochloris halophila and, therefore, we provisionally assigned our isolate to the genus Desmochloris. However, a close relationship of strain SAG 2397 with both D. halophila strains was not supported in significance tests and Desmochloris was paraphyletic in our 18S rRNA gene phylogeny (Figure 4). Consequently, assignment of strain SAG 2397 to a certain species within the subclade of Ulvales remained uncertain.

Discussion

Distribution of the Isolates at the Sampling Sites

In total, we detected nearly the same numbers of green algal species in the Deinschwanger (DB; 21) and Westerhöfer (WB; 18) creeks, regardless of the unequal number of sites sampled per creek (8 at the DB, 5 at the WB; Table 1). With respect to calcification the various sampling sites of the DB did not cluster according to high or low calcification when using their species compositions (Figure 8). We conclude that we observed a rather accidental distribution of the biofilm microalgae which is not or only very little influenced by calcification. This is contrast to findings of Brinkmann et al. (this issue) who reported the diversity of cyanobacteria and diatoms of the same creek biofilms as studied here was clearly influenced by $\text{SI}_{\text{calcite}}$ and $p\text{CO}_2$, which are reciprocally linked.

The highest diversity per site (11 species) was detected at site DB6, which was highly calcified (Table 1). However, from the other calcified sampling sites (DB1-DB5) only 1–3 species per site could be retrieved in culture. At the spring site DBS with calcification almost absent also just four species were recovered. Two of them, Coccomyxa cf. pringsheimii and Dilabilifilum printzii were retrieved only at the DB spring site, but D. printzii was recovered from the highly calcified sites of the WB as well (Table 1).

In addition, we compared the various DB sites for their “phylogenetic diversity,” i.e., whether the species recovered from a certain DB site belong to a single or several phylogenetically distinct clades. With respect to the number of phylogenetic clades retrieved per site, DB6 again was the most diverse site and the spring site DBS was the second-most diverse site (Table 2). The Chlamydomonadales clade (Chlorophycaceae; Figure 2) seemed to have a preference towards the highly calcified DB sites, whereas the Chlorel-lales (Trebouxiophycaceae; Figure 1) and the Ulotrichales (Ulvophyceae; Figure 4) clades were found at the highly calcified sites as well as the spring site of the DB (Table 2). With respect to the three different green algal classes detected in both karst-water creeks, the WB was dominated by members of Trebouxiophycaceae (12 out of the total of 18 species detected there belonged to Trebouxiophycaceae), whereas the DB predominately harbored members of Chlorophycaceae (12 out of the total of 21 species detected there belonged to Chlorophycaceae).

For the few members of Ulvophyceae no preference to any of the both creeks was found. Interestingly, out of the 34 detected green algal species only six were retrieved from both creeks, i.e., Coccomyxa cf. pringsheimii, Muriella terrestris and Stichococcus sp.1 of the Trebouxiophycaceae, Chlorococcum ellipsoideum-relative2 of the Chlorophycaceae, and Dilabilifilum printzii and Pseudendocloniopsis botryoides of the Ulvophyceae (Figure 9). It follows that the species richness of green algae at both creeks was rather different (Figure 9).

Species Identification and Origins

The molecular phylogenetic diversity of inland freshwater green algae from running waters has been studied for the first time here. In particular, for estimating the diversity of microscopic green algae in epilithic biofilms of running freshwaters, studies are rare and all were exclusively based on morphology so far (for review see Lindstrom et al. 2004; Veselá 2006). The majority of existing evidence about the molecular diversity of
freshwater green microalgae refers to communities rather different from algal biofilms, e.g., phytoplankton (Fawley et al. 2004; Krienitz and Bock 2012). Other molecular data for the diversity of green algae from of running freshwaters have mainly been available from habitats with extreme environmental variables or artificial impact (e.g., Aguilera et al. 2007; Aguilera et al. 2010; Baker et al. 2009; Dorigo et al. 2002; Palacios et al. 2008). For example, molecular data are available for the periphyton of the extremely cold and remote Antarctica (e.g., De Wever et al. 2009; Vyverman et al. 2010). Compared to freshwater creeks, terrestrial habitats have more extensively been studied by a combined morphological/molecular approach to date (e.g., Flechtner et al. 2013; Kulichová et al. 2014; Lewis and Lewis 2005).

Sequence comparisons of 18S rRNA genes enable the unambiguous comparisons of new isolates to those previously isolated from other localities and habitats world-wide (De Wever et al. 2009; Hodač et al. 2012; Němcová et al. 2011). Using sequence comparisons most of our isolates were closely related to those of various other inland freshwaters (Supplementary Table 3). Within the Trebouxiophyceae, these were members of the Chlorellales clade, i.e., Chlorella (Bock et al. 2010; Bock et al. 2011), Muriella (Fawley et al. 2004) and Marvania (Fawley et al. 2004; Yamamoto et al. 2007). Within the Chlorophyceae, at least the Scenedesmaceae, Selenastraceae and Mychonastes, which we also retrieved in our study, are well known from freshwaters (Krienitz et al. 2011, Krienitz and Bock 2012).

However, all these genera and families of green algae are known predominantly from major water bodies like lakes or ponds where they inhabit free-floating communities (phytoplankton) and, therefore, were not expected to occur in biofilm assemblages as well. Although Trebouxiophyceae and many Chlorophyceae are predominantly found in terrestrial and freshwater habitats, Ulvophyceae is a green algal class with a main divergence in marine habitats; it represents the only lineage of Chlorophyta that includes macroscopic seaweeds (Leliaert et al. 2012).

In contrast, our isolates were nested within the orders Ulotrichales and Ulvales, which are known to occur also in freshwaters and terrestrial habitats as well. Interestingly, the characteristically branched filamentous thalli of the majority of our ulvophyccean isolates strongly resembles the morphology of Gongrosira, which has frequently been reported from tufa formations (e.g., Freytet and Plet 1991; Johnson and John 1992; Pentecost 1988; Pentecost and Spiro 1990). Therefore, the name Gongrosira may represent a collective morphotype whose members are distributed at least on several distant lineages within the Ulotrichales (Ulvophyceae). Moreover, the only so far
habitats. Species of Coccomyxa SAG 2406 (Stichococcus and G) have been reported only from terrestrial habitats (Ettl et al. 2007). The latter three examples indicate that at least parts of the phytoplankton so far, but were recovered from creek biofilms attached to stones in running waters for the first time. In other cases closest relatives to our isolates were from terrestrial habitats. Our finding of E. subsphaerica is the first record of this species from a freshwater habitat; previously it has been reported only from terrestrial habitats (Ettl and Gartner 1995). Similarly, our still unidentified isolates of Stichococcus WB38 (S. cf. deasonii), SAG 2408 (S. sp.3) and SAG 2406 (S. sp.4), have their closest relatives from terrestrial habitats. Species of Coccomyxa have mostly been reported from terrestrial habitats (e.g., tree bark or lichen symbiosis) so far, but were recovered from creek biofilm in our study. The latter three examples indicate that at least parts of the creek biofilm algal communities were probably driven into their habitat through (rain-) flushes from the neighboring soils. For most other biofilm species which so far were reported from phytoplankton only it is still not known whether they can be found in terrestrial habitats as well. Muriella terrestris may be an example for a green microalgal species that is known from phytoplankton as well as soil; it was frequently found in the creek biofilms as well.

For 24 species we recovered no identification at the species level was possible, i.e. they were left unnamed despite they could be assigned to a certain genus in most cases. These represent species for which no 18S rRNA gene sequence have become available yet or which have been retrieved for the first time. In this respect we see the importance of molecular-phylogenetic investigations of inland freshwater algal biodiversity, i.e., to extend our knowledge about the distribution of species of green microalgae across various freshwater and terrestrial habitats.

Acknowledgments

We are indebted to Dr. Anke Behnke and Ms. Viviane Kipp who initiated this study through their culturing efforts and developed green algal PCR primer LR1650.

Funding

This work was supported by the German Science Foundation (DFG) by a grant extended to T.F. (Fr 905/13-2). Parts of this work were also supported by the German Federal Ministry of Education and Research, BMBF (AlgaTerra project, grant 01 LC 0026) within the BIOLOG program.

Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

References

Aguilera A, Zettler E, Gomez F, Amaral-Zettler L, Rodriguez N, Amils R. 2007. Distribution and seasonal variability in the benthic eukaryotic community of Rio Tinto (SW, Spain), an acidic, high metal extreme environment. System Appl Microbiol 30:531–546.

Aguilera A, Souza-Egipsy V, Gonzalez-Toril E, Rendueles O, Amils R. 2010. Eukaryotic microbial diversity of phototrophic microbial mats in two Icelandic geothermal hot springs. Inter Microbiol 13:21–32.

Altschul SF, Madden TL, Schaffer AA. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25(17):3389–3402.

Arp G, Bisset A, Brinkmann N, Cousin S, De Beer D, Friedl T, Mohr KL, Neu TR, Reimer A, Shiraishi F, Sticklandt E, Zippel B. 2010. Tufa-forming biofilms of German karstwater streams: microorganisms, exopolymers, hydrochemistry and calcification. Geol Soc Lond Special Publ 336: 83–118.

Baker BJ, Tyson GW, Goosherst L, Banfield JF. 2009. Insights into the diversity of eukaryotes in acid mine drainage biofilm communities. Appl Environ Microbiol 75(7):2192–2199.

Bilan MI, Usov AI. 2001. Polysaccharides of calcareous algae and their effect on the calcification process. Russ J Biochem 27:2–16.

Bock C, Krienitz L, Proschold T. 2011. Taxonomic reassessment of the genus Chlorella (Trebouxiophyceae) using molecular signatures (barcodes), including description of seven new species. Fottea 11(2):293–312.

Bock C, Proschold T, Krienitz L. 2010. Two new Dictyosphaerium-morphotype lineages of the Chlorellaceae (Trebouxiophyceae): Heynigia gen. nov. and Hindakta gen. nov. European J Phycol 45(3):267–277.

Brinkmann N, Hodaç L, Mohr KL, Hodaçová A, Jahn R, Ramm J, Hallmann C, Arp G, Friedl T. Submitted. Cyanobacteria and diatoms in biofilms of two karstic streams in Germany and changes of their communities along calcite saturation gradients. Geomicrobiol J, this issue.

De Wever A, Leliart F, Verleyen E, Vanompeninghen P, Van der Gucht K, Hodgson DA, Sabbe K, Vyverman W. 2009. Hidden levels of phylodiversity in Antarctic green algae: further evidence for the existence of glacial refugia. Proc Roy Soc B: Biol Sci 276(1673):3591–3599.

Dorigo U, Béard A, Humbert JF. 2002. Comparison of Eukaryotic phytoebenthic community composition in a polluted river by partial 18S rRNA gene cloning and sequencing. Microb Ecol 44:372–380.

Ettl H, Gärtner G. 1995. Syllabus der Boden-, Luft-, und Flechtenalgen. Stuttgart: Gustav Fischer Verlag.

Fawley MW, Fawley KP, Buchheim MA. 2004. Molecular diversity among communities of freshwater microchlorophytes. Microb Ecol 48:489–499.
