Biodiversity of terrestrial algal communities from soil and air-exposed substrates using a molecular approach

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General Introduction

Eukaryotic microalgae are widespread phototrophic organisms not only in aqueous habitats like freshwater or the marine environment, but are also common and abundant in diverse terrestrial habitats (Hoffmann, 1989; López-Bautista et al., 2007; Karsten et al., 2007; Rindi et al., 2010; Holzinger and Karsten, 2013; Ettl and Gärtner, 2014). Among terrestrial algae, the green algae, along with the prokaryotic cyanobacteria, may be the most diverse group (Hoffmann, 1989; Rindi, 2007; Rindi, 2011). The eukaryotic terrestrial green algae comprise both phyla Chlorophyta and Streptophyta (Lewis and McCourt, 2004; Pröschold and Leliaert, 2007). Terrestrial green algae are particularly diverse in the Chlorophyta where they are distributed on at least three lineages, the classes Trebouxiophyceae, Chlorophyceae and Ulvophyceae (Friedl and Rybalka, 2012; Leliaert et al., 2012). Out of these three classes the Trebouxiophyceae appear to comprise predominantly terrestrial algae as most of its members are known from dry habitats exposed to air (aeroterrestrial algae), soil or lichen symbiosis (Friedl and Rybalka, 2012; Leliaert et al., 2012). With respect to colonization of land the Streptophyta may have been the most successful lineage, because the embryophytes have their origin within the Streptophyta (Karol et al., 2001; Turmel et al., 2007; Wodniok et al., 2011). Common terrestrial algal groups of the Streptophyta are members of the Klebsormidiophyceae (Rindi et al., 2011; Ryšánek et al., 2015). In general, several independent origins of "land plants", i.e. terrestrial green algae have been discussed (Lewis and Lewis, 2005; Lewis, 2007).

Terrestrial algae are obvious at almost all air exposed substrates, e.g. rocks, building stone, monument surfaces (Crispim et al., 2003; Hoppert et al., 2004; Macedo et al., 2009), artificial substrates in urban environment (such as house facades and roof tiles; Karsten et al., 2007), tree barks (Gärtner, 1994; Lüttge and Büdel, 2010; Neustupa and Štifterová, 2013) where green algae often form conspicuous green phototrophic biofilms or crusts, as well as soils. The green algal communities (as well as the cyanobacterial communities) on stone surfaces may be involved in biodeterioration of their substrates growing epi- and endolithically on rocks as well as building material and monument surfaces (Saiz-Jimenez, 1995; Warscheid and Braams, 2000; Crispim et al., 2003; Gaylarde et al., 2003; Hoppert et al., 2005; López-Bautista et al., 2007; Cutler et al., 2013). They also contribute to undesirable coloration observed in urban habitats on various man-made substrates, e.g. building facades and roof tiles (Barberousse et al., 2006; Rindi, 2007; Gladis-Schmacka

et al., 2014). Soils are the most important non-aqueous habitats for terrestrial algal communities (Zenova et al., 1995). They have beneficial functions in soil, e.g. provide organic matter as food source, perform nitrogen fixation, and interact with higher plants (Metting, 1981; Starks et al., 1981). These green algal communities together with cyanobacteria are among the first organisms colonizing bare soils, deserts or disturbed sites and promote soil stability and constitute water-stable aggregates to prevent erosion (Johansen, 1993; Lewis and Lewis, 2005; Büdel et al., 2014).

Traditionally, the composition of terrestrial green algal communities have been assessed by the establishment of enrichment cultures followed by the development of pure unialgal cultures. These isolates have been identified by microscopic features which, however, are often rather spare in distinctive characters which may cause uncertainties in the identification. Only single terrestrial green algal isolates have been identified at the molecular level, i.e. by comparisons of DNA sequence signatures which enable an unambiguous identification and re-identification of algal species. Using traditional culture methods only, there have been considerable uncertainties in the diversity of terrestrial green algal communities due to different identification strategies depending on the authors and taxonomic uncertainties of previously published floristic lists (Hoffmann, 1989; Lukešová and Hoffmann, 1996; Neustupa and Škaloud, 2010). A culture-independent molecular approach provides unambiguous identification of green algal communities e.g. in terrestrial environments. Even if the recovered species may not be exactly named in case of lack of appropriate reference sequences or taxonomic problems, the algal taxa can be unambiguously recovered by their unique molecular signature. This enables not only a more reliable approach to determine the diversity of an algal community, but also permits comparisons among the algal communities of the same and different habitats.

Aim of the thesis

The main objective of this thesis was to test the reliability of a molecular approach for the diversity assessment of terrestrial green algal communities using several exemplar habitats, i.e. open air-exposed-ones as well as soil. It should be further evaluated in as much the green algal species composition can be unambiguously determined so that comparisons of different communities at the same habitat/substrate along a gradient of abiotic parameters or between different habitats and substrates are possible. 18S rRNA gene sequences were used determined from environmental clone libraries. This approach was compared with corresponding analyses based on enrichment cultures or unialgal isolates in selected cases, i.e. chapters 4 and 6.

Chapter 1 describes the diversity of air-exposed phototrophic biofilms, including cyanobacteria, on natural building stone. The communities of two wall areas with different expositions towards sun were compared. **Chapter 2** and **Chapter 3** present the assessment of biofilms consisting green algae and fungi covering stone monuments, their composition and potential involvement in biodeterioration. Additionally, the re-colonization after cleaning and restauration procedures were investigated. The analysis of a green algal biofilm on an exemplar artificial hard substrate in an urban environment is outlined in **Chapter 4.** In **Chapter 5** also a natural air-exposed substrate, different types of tree bark, was studied in the framework of the German Biodiversity Exploratories, a large scale research project which attempts to uncover effects of management types and intensities of biodiversity. Finally and also in frame of the latter project, in **Chapter 6**, the green algal diversities of different forest and grassland soils from defined research plots at three different geographic regions in Germany were compared.

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Chapter 1:
Molecular diversity of phototrophic biofilms on building stone

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Abstract

Composition and diversity of aeroterrestrial phototrophic microbial communities are up to now poorly understood. Here, we present a comparative study addressing the composition of algal communities on sandstone substrata based upon the analysis of rRNA gene clone libraries from environmental samples and crude cultures. From a west-facing, shaded wall area of the mediaeval castle ruin Gleichen (Thuringia, Germany), sequences mainly related to the green algae *Prasiococcus* and *Trebouxia* (Trebouxiophyceae) were retrieved. A south-west-facing, sun-exposed wall area was mainly colonized by *Apatococcus* and a *Phyllosiphon*-related alga. Just a few species, in particular *Stichococcus*-related strains, were ubiquitous in both areas. Samples from a basement vault exposed to low irradiance exhibited Chlorophyceae like *Chromochloris* and *Bracteacoccus*. Thus, most green algae on the daylight-exposed walls were affiliated to Trebouxiophyceae, whereas Chlorophyceae were dominant in samples taken from the site kept under low irradiance. Accordingly, cyanobacterial communities were different: the sun-exposed area was dominated by *Synechococcus*-related organisms, while on the shaded wall area, cyanobacteria were almost absent. The filamentous *Leptolyngbya* dominated samples from the basement vault. Scanning electron microscopy revealed endolithic algal morphotypes (coccoid algae and diatoms) dominant in open pores between mineral particles. Here, the organisms may be also involved in biogenic weathering of stone.

Keywords: green algae, cyanobacteria, environmental sample, 18S rRNA gene, scanning electron microscopy.

Introduction

Phototrophic microorganisms are important primary producers on hard rock substrata as well as on building facades (e.g. Karsten et al., 2007a; Horath and Bachofen, 2009). Eukaryotic microalgae and cyanobacteria, along with fungi and lichens, have also been recognized as important factors for rock weathering and stone decay (e.g. Welton et al., 2003; Büdel et al., 2004; Gorbushina, 2007). The rock substratum itself mostly provides harsh environmental conditions. Temperature may vary by several tens of degrees Centigrade during a day, accompanied by rapid desiccation (or freezing); that is, the availability of water is extremely limited (e.g. Walker and Pace, 2007). The availability of nitrogen and sulphur compounds as well as phosphate strongly depends on the substratum and other nutrient sources in the immediate surrounding, for example precipitation, soil, bird droppings and volatile emissions (e.g. Karsten et al., 2003).

It has been recognized that in extreme habitats, the productivity of the organisms must be close to lowest possible limits (e.g. Johnston and Vestal, 1991). Despite adverse conditions, the mineral substrata may be colonized on the surface (epilithic) or inside the substratum (endolithic) by relatively highly diverse communities of phototrophic and heterotrophic microorganisms (mainly fungi and bacteria).

Several studies identifying algae according to their morphology reported differences in algal diversity depending on diverse substrata including stone (e.g. Bellinzoni et al., 2003; Crispim et al., 2003; Uher, 2008; Macedo et al., 2009; Khaybullina et al., 2010). However, so far only few studies based on a culture-independent approach using rRNA gene as phylogenetic marker were performed for mineral substrata (e.g. Horath and Bachofen, 2009; Cuzman et al., 2010; Ragon et al., 2012). Also knowledge on factors that may determine the algal diversity on different but closely neighbouring sites is still lacking. It is obvious that - apart from irradiance - composition of the algal communities is determined by other physical parameters as well. In soil, the pH appears to be one factor that influences the dominance of the major groups of photoautotrophic organisms: cyanobacteria are known to prefer neutral and alkaline soils (Shields and Durell, 1964; Brock, 1973), whereas green algae prefer acidic soils (Starks et al., 1981; Lukešová and Hoffmann, 1995). In addition, cyanobacterial and green algal communities in soil may be influenced by soil type (Garcia-Pichel et al., 2001) and land use (Zancan et al., 2006). Previous studies of phototrophic communities on various building stones and rock substrata demonstrated that nutrients have less influence on the community structure than,

for example, UV radiation, pH and aspect (Bellinzoni et al., 2003; Furey et al., 2007). Microalgal and cyanobacterial resting stages may easily resist adverse environmental conditions (e.g. Häubner et al., 2006; Lennon and Jones, 2011). These resting stages and other kinds of propagules may be present as a 'seed bank' (Lennon and Jones, 2011). They may turn active after environmental conditions change. Thus, it may be assumed that a multitude of organisms will be detectable and even culturable from habitats with adverse environmental conditions, despite these organisms may be inactive and just present in low numbers in their natural habitat.

In this study, we show that the composition of phototrophic microbial algal communities including cyanobacteria differed markedly between apparently similar substrata. This is mainly due to differences in the exposure to sunlight (and hence water availability) and the occurrence of gypsum crusts. We used a molecular approach that allowed the identification of microalgae including cyanobacteria down to generic or even species level. We also discuss the results in view of possible biodeterioration mechanisms.

Materials and methods

Sampling sites

The sampling sites were several wall sections of the castle Gleichen, near Gotha, Thuringia, Germany (50°52′49″N, 10°50′20″E). In this location, the average regional annual temperature is 8.1 °C, the sunshine duration is about 1500 h per year, and annual precipitation is about 560 mm (data taken from Deutscher Wetterdienst Offenbach, Germany). In the year of sampling (sampling date May 15, 2009), precipitation was higher than the long year average (March-May 173 mm, compared with 120 mm long year average in these months). Average temperatures in March (4.2 °C), April (11.2 °C) and May (13.2 °C) were slightly higher than the respective long year averages in the first two months (2.8 °C, 7.5 °C and 13.2 °C, respectively).

Samples were taken from two walls. A south-west-facing wall facade (wall area A; slope 90°, aspect value 210°) was exposed to direct sunlight for 8-10 h during these months. A west-facing facade (wall area B; slope 90°, aspect value 275°), due to the existing architectural structures, was reached by direct sunlight just in the afternoon for 3 h (from 4.00 h pm onwards, without direct sunlight in March). A third sampling site (basement vault) was not reached by direct sunlight. The walls consisted of various types of dimension stones (Stück et al., 2011; see below).

Sampling and cultivation

Samples of approximately 100 µl dry volume were collected in May 2009 from south-west-facing wall area A (Fig. 1a, b), west-facing wall area B (Fig. 1c, d) and a basement vault in a distance of 5 m from the entrance (Fig. 1e, f). The samples were scraped off with a sterile scalpel and collected in sterile 2 ml reaction tubes. Biofilm samples used for establishing the clone libraries were randomly taken from each wall area. All samples in area A were taken from sandstone (Gleichenberger Rhätsandstein) (c.f. Stück et al., 2011). Wall joints were mostly closed, that is, filled with gypsum mortar. Area B exhibited a variety of limestone and sandstone lithologies (travertine, Grenzdolomit, Rhätsandstein). Most wall joints were open. Again, just samples from sandstone (Rhätsandstein) were taken into consideration for this study. Samples from green biofilms grown in the inner faces of forming scales were collected from the basement vault (Fig. 1e-h).

For establishing crude cultures, aliquots of the biofilm samples were suspended in flasks with 20 ml 3N BBM+V medium (Starr and Zeikus, 1993) for green algae, BG11 medium (Rippka and Herdman, 1993) for cyanobacteria and Diat medium for diatoms (c.f. http://www.uni-goettingen. de/de/186449.html). The crude cultures were incubated at 18 °C on a 14:10 h light:dark cycle at 25 µmol photons m^{-2} s^{-1} from white fluorescent light for four weeks.

DNA extraction

General procedures for handling and examination of DNA were performed according to Sambrook et al. (2000). Genomic DNA was extracted from collected environmental biofilm samples and crude cultures. If applicable, cultures grown on 3N BBM+V or BG11 were mixed prior to DNA extraction. Cells were disrupted by shaking in a Mini-BeadBeater (Biospec Products, Bartlesville, OK) in the presence of equivalent amounts of acid-washed glass beads (120-200 µm and 425-600 µm in diameter; Sigma-Aldrich, St. Louis, MO) and vortexed briefly. The samples were treated in the bead beater for 30 s at 5.000 rpm. DNA was extracted with the Invisorb Spin Plant Mini Kit (STRATEC Molecular, Berlin, Germany), following the manufacturer's instructions.

The MoBio PowerSoil DNA isolation Kit (MoBio Laboratories Inc. Carlsbad, CA) was used for extraction of genomic DNA from samples of endolithic biofilms according to the manufacturer's instructions. Extraction results were evaluated after electrophoresis on a 1 % (w/v) agarose gel. Isolated DNA was stored at -20 °C until further processing.

Fig. 1. Sampling sites on Gleichen castle. (a) Wall area A, sun-exposed. (b) Wall A in detail, with wall joints mostly filled with mortar. (c) Shaded wall area B. (d) Wall area B in detail, most wall joints open. (e) and (f) Dark basement vault, with scale samples removed from the basement vault. (g) and (h) Scales with green biofilm. Black frames: sampling areas.

PCR amplification

For amplification of eukaryotic rRNA genes from DNA preparations, PCR was performed as follows: 18S rRNA genes were first amplified using eukaryotic specific primers 20F (5′ GTAGTCATATGCTTGTCTC 3′) and 18L (5′ CACCTACGGAAACCTTGTTACGACTT 3′; Hamby et al., 1988) followed by a second amplification (semi-nested PCR) with the primers 20F and the newly developed CH1750R (5′ CTTCCTCTARTGGGAGG 3′), complementary to positions 1734-1751 of the 18S rRNA gene sequence of *Chlorella vulgaris* SAG 211-11b (accession number X13688), specific for green algae (this study). For cyanobacteria from environmental samples and crude cultures, the primers PCR1 and PCR18 (Wilmotte et al., 1993) were used for amplification of 16S rRNA genes.

Approximately 30 ng of the extracted DNA was used as template in each amplification reaction. The reaction mixture (25 µl) contained each dNTP at a concentration of 0.1 mM, 2.5 µl of 10 x reaction buffer, 2 mM $MgCl_2$, each primer at a concentration of 0.2 µM, 2 U of Taq DNA polymerase (Bioline, Luckenwalde, Germany) and 4 % (v/v) dimethyl sulfoxide (DMSO) solution. PCR was performed in a thermocycler Primus 96^{Plus} (MWG-Biotech, Ebersberg, Germany) using the following programme for the primer set 20F/18L: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 3 min and final extension at 72 °C for 10 min. For the semi-nested PCR with the primer set 20F/CH1750R, a 1:25 dilution of the primary PCR product was used as template. The following programme was used: initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 3 min and final extension at 72 °C for 10 min. For the cyanobacterial primer set PCR1/PCR18, initial denaturation was at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 2 min and a final extension at 72 °C for 30 min.

From two crude cultures obtained from basement vault scales, diatom rRNA genes were amplified with specific primers as described in Pniewski et al. (2010). All PCR products were purified using the Invisorb DNA CleanUp Kit (STRATEC Molecular). Aliquots of 2 µl of the purified amplicons were analysed by electrophoresis on a 1 % (w/v) agarose gel.

rRNA gene cloning and sequencing

Cloning was carried out with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) with TOP 10 chemically competent One Shot *Escherichia coli* cells (Invitrogen), as supplied by the manufacturer. In the plasmid blue/white screening, white *E. coli* colonies containing correct DNA insertions were further identified by direct amplification of the inserted DNA fragment with a vector-specific primer set M13F/M13R (Invitrogen). Positive clones were cultivated overnight in 2 ml reaction tubes with 1 ml LB medium containing 100 µg ampicillin. Plasmid DNA was purified with the Invisorb Spin Plasmid Mini Two kit (STRATEC Molecular) and stored at -20 °C.

Sequencing reactions were performed with the Dye Terminator Cycle Sequencing v3.1 kit (Applied Biosystems, Darmstadt, Germany) and an ABI Prism 3100 (Applied Biosystems) automated sequencer. All eukaryotic clones were sequenced with the 18S standard sequencing primer 895R (5′ AAATCCAAGAATTTCACCTC 3′), resulting in partial sequences, including the hypervariable regions V2-V4 (Neefs and De Wachter, 1990; Hodač et al., 2012; Lee and Gutell, 2012). Prokaryotic clones were sequenced with PCR1 (Wilmotte et al., 1993), resulting in partial sequences including the hypervariable regions V1-V3 (Santamaria et al., 2012).

Phylogenetic and statistical analysis

The sequences were edited and assembled using SeqAssem (Hepperle, 2004). Sequences shorter than 400 bp were excluded from further analysis. The remaining sequences were compared with available sequences in NCBI by BLASTN (Altschul et al., 1990; http://www.ncbi.nlm.nih.gov/). Next, relative sequences were imported into the ARB program (Ludwig et al., 2004; http://www.arb-home.de). In addition, internal sequences provided by SAG Culture Collection of Algae (University of Göttingen) were included in the comparisons. To determine preliminary phylogenetic affiliations, the sequences were aligned with homologous rRNA gene sequences using the automatic alignment tool of the ARB program package.

Potential chimeras were checked by Bellerophon (Huber et al., 2004); in addition, the first and the last 300 bp of putative chimeras were compared with similar rRNA gene sequences in NCBI. Chimeric sequences were excluded from the data set.

Rarefaction curves and operational taxonomic units (OTUs) were calculated with MOTHUR (Schloss et al., 2009). OTUs were defined on the basis of ≥ 98 % sequence similarity for 18S and 16S rRNA gene sequences (Romari and Vaulot, 2004; Marande

et al., 2009; Michaud et al., 2012; Stock et al., 2012). Representative sequences of each OTU were selected and sequenced completely (Moon-van der Staay et al., 2001; Ragon et al., 2012) with standard sequencing primers. Representative sequences were deposited in GenBank under the following accession numbers: JX127160 - JX127192.

For phylogenetic analyses, alignments of rRNA gene sequences were performed using MAFFT, version 6 (Katoh and Toh, 2008), and small corrections were made by eye. Complete rRNA gene sequences were subjected to phylogenetic analyses using the maximum likelihood (ML) method by RAxML (Stamatakis et al., 2008), in conjunction with the GTR+Γ+I model with 100 bootstrap replicates. In addition, Bayesian posterior probabilities (MB) were calculated with MrBayes 3.2 (Huelsenbeck and Ronquist, 2001). Two parallel Markov chain Monte Carlo (MCMC) runs for two million generations each with one cold and three heated chains were conducted using the GTR+Γ+I model, with trees sampled every 100 generations.

To quantify differences between groups of samples, SIMPER (Similarity percentages) analysis was conducted using the program PAST, version 1.98 (Hammer et al., 2001). A similarity matrix was calculated based on the abundances (in percentages) of the algal OTUs. As a similarity measure, Bray-Curtis distance index was used. The significance of differences was tested by one-way ANOSIM using the same similarity measure.

Light and electron microscopy

Light microscopic observations were performed using an Olympus BX60 microscope (Tokyo, Japan) with Nomarski DIC optics equipped with a ColorView III camera (Soft Imaging Systems, Münster, Germany). Micrographs were processed using the Cell^D image software (Soft Imaging Systems).

For scanning electron microscopy (SEM), samples were fixed immediately after sampling in 2 % (w/v) glutardialdehyde (EM grade, Sigma-Aldrich, Deisenhofen, Germany) and stored at 4 °C until further processing. Samples were dehydrated in an ascending ethanol series (15-99 %), mounted on SEM sample holders and sputtered with Au-Pd (7.3 nm for 120 s). Samples were visualized in a SEM LEO 1530 Gemini (Zeiss, Oberkochen, Germany) combined with an INCA X-ACT EDX. Electron micrographs were colorized with Hornil StylePix (www.hornil. com/en/products/stylepix). Methods for transmission electron microscopy were performed as described in Hallmann et al. (2011a).

Results

Macro- and microscopic observations

For this study, samples were taken from two south-west- and west-facing wall areas (A and B) and from scales formed on sandstone on a basement vault. All sampled sites exhibited obvious colonization by cryptogams, including algal ('green') biofilms. Thalli of endolithic lichens were abundant on sandstone at the base of wall area A. In addition, a thin gypsum crust (W. Wedekind, personal communication) covered small sections of wall area A. Green biofilms were occasionally found under these crusts. On wall area B, thalli of crustose lichens were also present, but endolithic lichens were not observed. Along the basement vault, thick green coverings in the inner faces of scales (Fig. 1g, h) were observed.

SEM of the fracture faces of the scales from the basement vault revealed a dense cover by either filamentous (Fig. 2a and inset) or coccoid (Fig. 2b) morphotypes and diatoms (Fig. 2c, d). The coccoid cells were located in open pores and between small chips of the mineral particles (clay particles intermixed with gypsum according to EDX analysis). The filamentous morphotypes (putatively cyanobacteria, see below) built up a dense biofilm on the mineral particles (Fig. 2a).

Diatoms were identified as *Diadesmis contenta* D.G. Mann (Fig. 2c) and *Achnanthidium minutissimum* Czarnecki (Fig. 2d) (Krammer and Lange-Bertalot, 1986-2004). Additional diatom phylotypes were retrieved from two crude cultures by rRNA gene analysis (see below). Various morphotypes of green algae were detected by light microscopy of the crude cultures, for example the cell package-forming green alga *Prasiococcus calcarius* (Fig. 3a). Also filamentous cyanobacteria were observed in these cultures (Fig. 3b).

rRNA gene analysis of the phototrophic community

Analyses of 16S and 18S rRNA genes aiming at detection of eukaryotic algae and cyanobacteria were performed for environmental samples and for crude cultures. Enrichment in crude cultures allows detection of organisms that are present in the original sample in just extremely low numbers of individuals, which may leave these species undiscovered in the environmental biofilm.

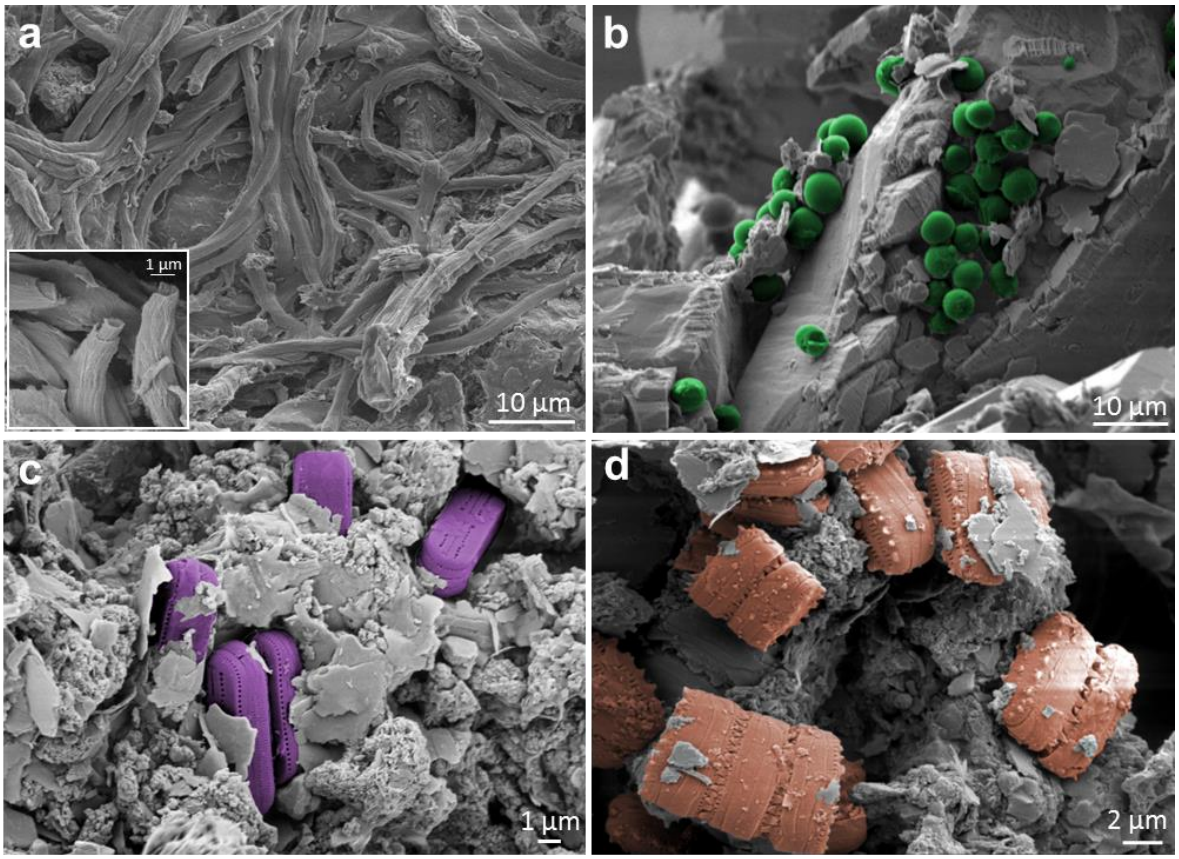

Fig. 2. Scanning electron microscopy images of scales as depicted in Fig. 1h. (a) Filamentous morphotypes cover the inner face of a scale as a dense biofilm. Inset: sheathed filaments in close-up. (b) Coccoid algal cells between small chips (green pseudocolor). (c) Diatoms (*Diadesmis contenta*) located in open pores (magenta pseudocolor). (d) Chains of the diatom *Achnanthidium minutissimum* (orange pseudocolor).

Cyanobacteria

16S rRNA gene clone libraries were established from four environmental samples of wall area A and six of wall B. Four clone libraries from basement vault scale samples were established from crude cultures.

A total of 11 cyanobacterial OTUs were recovered. With respect to cyanobacteria, wall areas A and B differed markedly (Fig. 4). On wall area A, 47 of 75 clones were assigned to cyanobacteria, with the majority of them (46) representing *Synechococcus*-like OTUs (cyanobacterial OTU 3 and 11, Table 1, Fig. 4). On wall area B, just seven of 70 sequenced clones were represented by cyanobacteria.

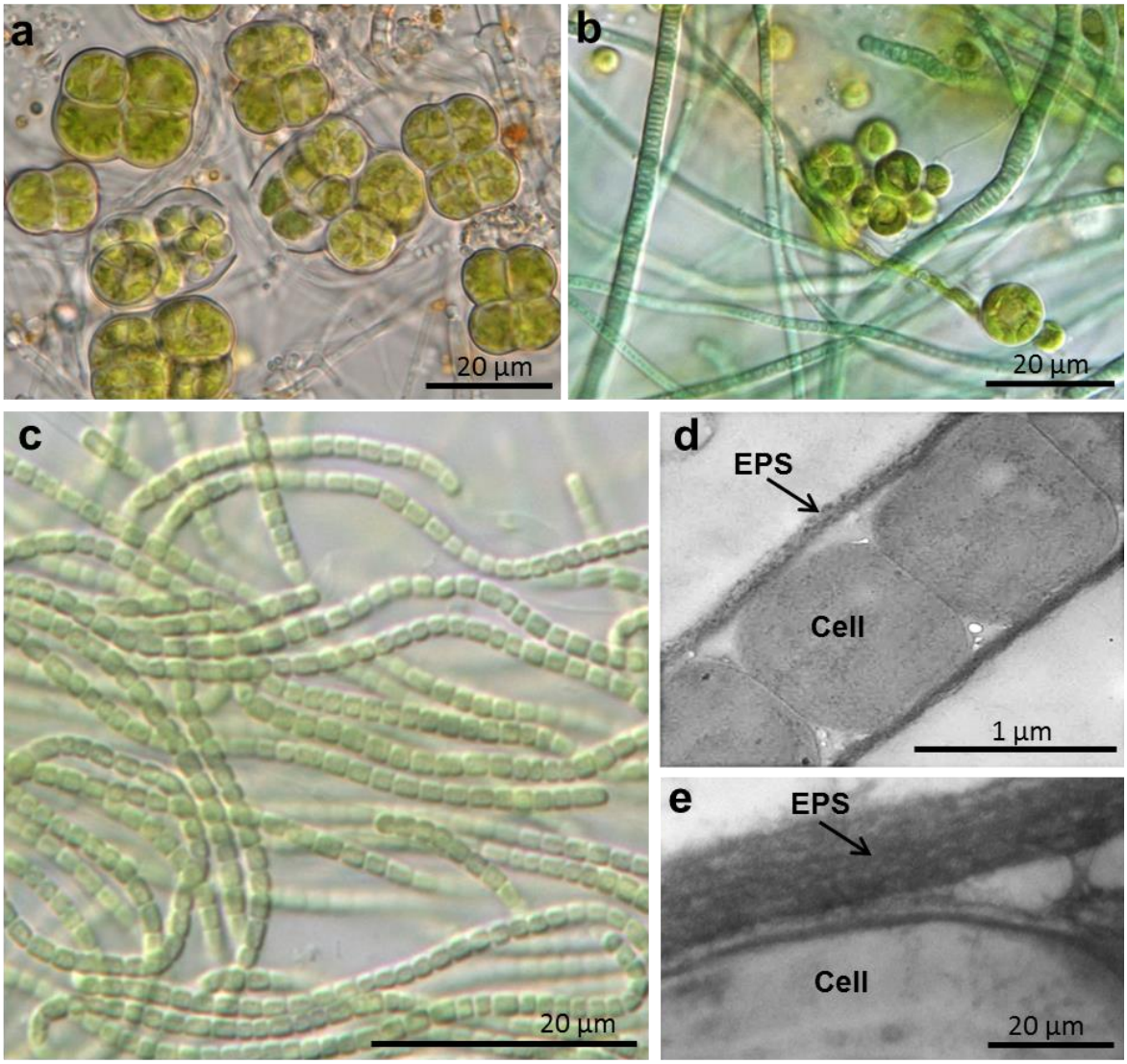

Fig. 3. Different morphotypes of cyanobacteria and green algae. (a) Crude culture with cell packages of *Prasiococcus calcarius*. (b) Crude culture with green algae and filamentous cyanobacteria. (c) Light micrograph of a *Leptolyngbya* sp. isolate. (d) and (e) Electron micrographs of a filament of *Leptolyngbya*, obtained by transmission electron microscopy of ultrathin sections with a multilayered exopolysaccharide (EPS) sheath; close-up view in (e).

From basement vault scale samples (Fig. 1g, h), 14 clones were retrieved, but with a dominance of clones representing *Leptolyngbya*-like cyanobacterial OTU 2 (Fig. 3c). This was in accordance with a high frequency of sheathed filamentous cyanobacteria as observed by electron microscopy of respective samples (Fig. 3d, e). In addition, three OTUs could be assigned to uncultured cyanobacteria (OTU 4, 5 and 6), three OTUs related to *Chroococcus*, one OTU related to *Microcoleus vaginatus* and one OTU representing *Leptolyngbya*-like cyanobacterial OTU 1 (Table 1, Fig. 5).

Table 1. Distribution of cyanobacterial OTUs on different wall areas.

	No. of clones					
OTU 98 %	Wall A	Wall B	Scale	Representative clone	Acc. No.	Closest relative (% similarity)
1		1		3GB21_K52	JX127187	*Leptolyngbya* sp. ANT.L52.1, AY493584 (99 %)*
2			10	3GSCS_K18*	JX127186	*Leptolyngbya frigida* strain ANT.LMA.1, AY493573 (96 %)
3	41	1	1	3GSG3R_K29*	JX127188	Uncultured cyanobacterium clone GG3, JQ404415 (96 %), *Synechococcus* -related
4			3	3GSCR_K16*	JX127185	Uncultured bacterium clone AK4AB1_05A, GQ396895 (99 %), *Chroococcodiopsis* -related
5		1		3GB20_K48	JX127184	Uncultured bacterium clone 139, HM241004 (96 %)*, *Chroococcodiopsis* -related
6	1			3GA1-12_K100	JX127183	Uncultured cyanobacterium clone F3Baug.33, GQ417856 (99 %)*, *Chroococcodiopsis* -related
7		1		3GB13_K125	JX127182	*Microcoleus vaginatus* isolate UBI-KK2, EF654079 (100 %)*
8		1		3GB1-7_K19	JX127190	*Chroococcus* sp. strain 2T05h, FR798926 (97 %)*
9		1		3GB1-7_K20	JX127192	*Chroococcus* sp. strain 2T05h, FR798926 (92 %)
10		1		3GB1-7_K18	JX127191	*Chroococcus* sp. strain 2T05h, FR798926 (96 %)
11	5			3GA1-12_K89	JX127189	Uncultured cyanobacterium clone GG3, JQ404415 (98 %)*, *Synechococcus* -related

*Representative full length sequence in phylogenetic analysis.

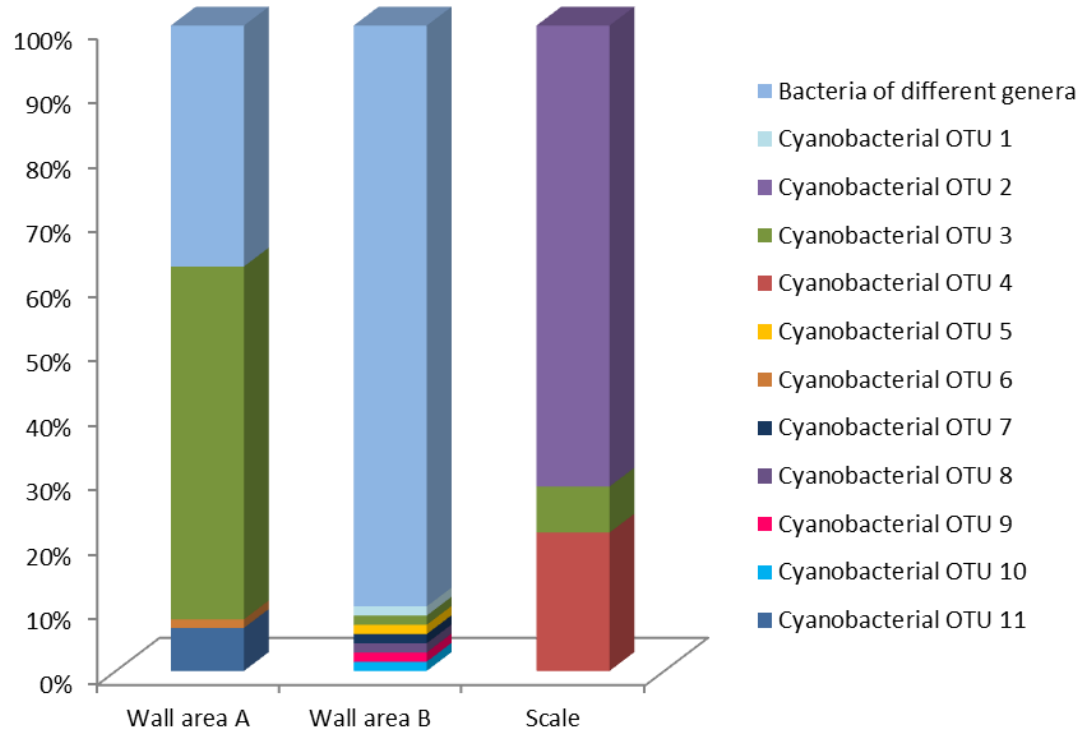

Fig. 4. Relative abundance of cyanobacterial operational taxonomic units (OTUs; 98 % cut-off), obtained after analysis of 16S rRNA gene sequences, reveals clear differences between the south-west-facing wall area A, the west-facing wall area B and scale samples from the basement vault.

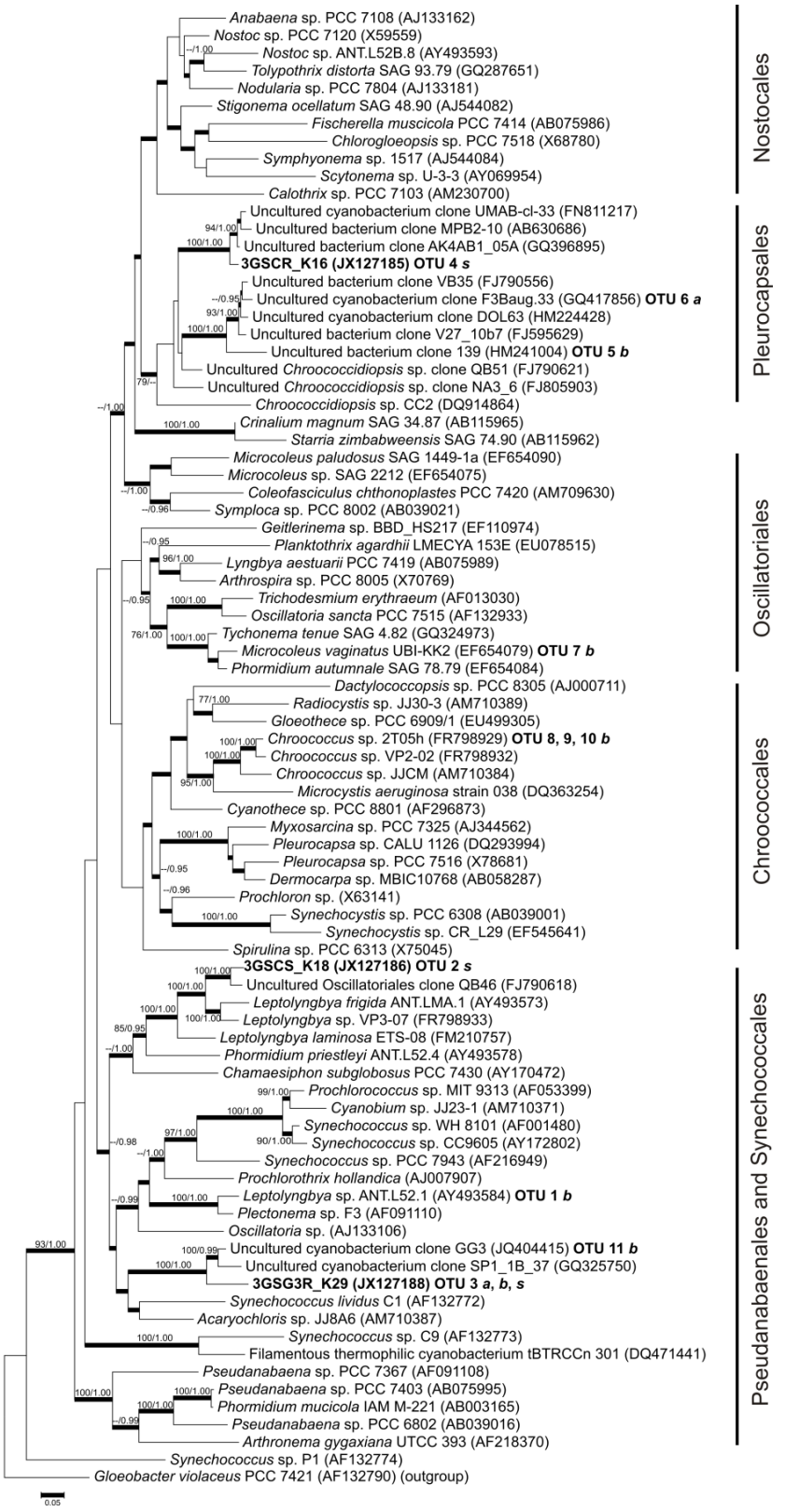

Fig. 5. Phylogenetic tree of cyanobacteria based on 16S rRNA gene sequences. Maximum likelihood method (RAxML) based on 84 taxa and 1490 positions. Thick lines indicate internal nodes that were received in maximum likelihood and Bayesian trees. Support values given as ML (maximum likelihood; > 75 % bootstrap values)/MB (MrBayes; > 0.95 Bayesian posterior probabilities). Sequences in bold represent full 16S rRNA gene sequences of clones; a: wall A; b: wall B, s: scale.

Green Algae

DNA preparations from environmental samples and from crude cultures were analysed with general eukaryotic primers and specific green algal primers as described in the methods section. In total, 648 18S rRNA partial sequences including 482 green algal clones were recovered from all sampling sites. These resulted in 22 green algal OTUs at a cut-off of 98 % sequence homologies (Table 2).

Rarefaction curves (Fig. 6) calculated for the biofilm samples of wall areas A and B and the basement vault scale samples reveal the clone library coverage for algal sequences. For wall area A, the rarefaction curve reached almost a plateau, and for wall area B, nearly full coverage of OTUs was reached. For the scale samples (biofilm samples and crude cultures), saturation was not reached.

On wall area A, 149 green algal clones representing 11 OTUs and eight fungal clones were found. Samples from wall area B yielded in 253 green algal clones representing 10 OTUs; 24 clones represented fungi. From scales, 214 clones were established, which were distributed on green algae (80 clones) representing 12 OTUs, flagellates (11 clones), fungi (13 clones) and mosses (110 clones). The green algal clones were processed for further statistical and phylogenetic analyses.

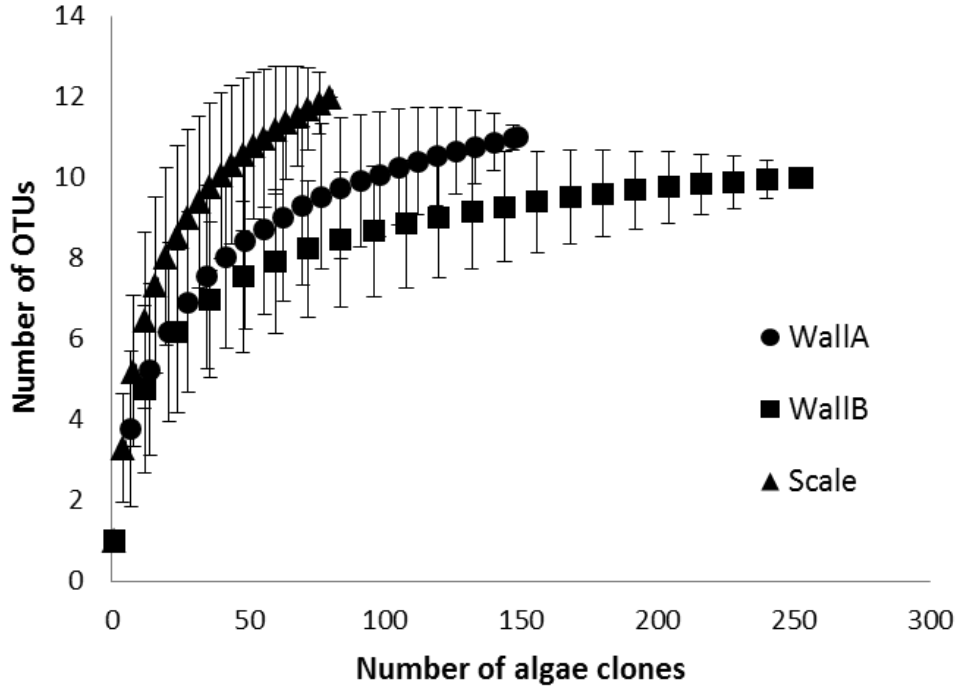

Fig. 6. Rarefaction curves determined for 18S rRNA gene clone libraries. The OTUs include the green algal sequences from both wall areas (A and B) and from scale samples.

Table 2. Distribution of green algal OTUs on different wall areas.

		No. of clones						
OTU	98 %	Wall A	Wall B	Scale	Representative clone	Acc. No.	Closest relative (% similarity)	
1		23		1	3GSCRE_K20*	JX127160	Uncultured eukaryote clone Th090408_11, HM030915 (99 %), *Apatococcus* -related	
2			2		3GB1314RE_K32	JX127170	*Pabia signensis* strain SAG 7.90, AJ416108 (99 %)*	
3		27	94		3GB18_K125*	JX127161	*Stichococcus mirabilis* CCAP 379/3, AJ311638 (99 %)/*Pseudostichococcus monallantoides*	
4		8	18	13	3GSG1RE_K41*	JX127162	*Stichococcus minutus* strain NJ-17, JN400256 (9 9%)	
5			3		3GB14_K3762*	JX127167	*Stichococcus* sp. strain MBIC10465, AB183601 (98 %)	
6			76		3GB14_K3784*	JX127166	*Prasiococcus calcarius* isolate Prca1 (SAG 10.95), EF200527 (99 %)	
7			11		3GB22_K3884	JX127169	*Rosenvingiella* sp. GALW014367, EF200523 (99 %)*	
8		9	15		3GB17_K62*	JX127168	*Stichococcus* sp. strain MBIC10465, AB183601 (99 %)	
9				3	3GSG3RE_K13*	JX127163	*Myrmecia bisecta* strain IB T74, Z47209 (99 %)	
10		3		7	3GSG3RE_K25*	JX127164	*Chlorella vulgaris* SAG 211-11b, X13688 (99 %)	
11		1		1	3GSG3R_K40*	JX127165	*Nannochloris bacillaris* , AB080300 (99 %)	
12			1		3GB19_K3848	JX127174	*Elliptochloris* sp. SAG 2117, FJ648515 (99 %)*	
13		1	27		3GB13_K3760*	JX127171	*Trebouxia asymmetrica* SAG 48.88, Z21553 (98 %)	
14		5			3GA1-4_K3642	JX127172	Uncultured Trebouxiophyceae clone QE59, FJ790667 (99 %)*, *Trebouxia* -related	
15		2			3GA1-4_K3641	JX127173	Uncultured Trebouxiophyceae clone QE59, FJ790667 (98 %)*, *Trebouxia* -related	
16		67	6	1	3GB14_K3808*	JX127175	'*Phyllosiphon arisari* '-isolate PY9a1, JF304471 (99 %)	Trebouxiophyceae
17				8	3GSG6RE_K19	JX127181	*Bracteacoccus* sp. UT8-26, AF513376 (99 %)*	
18				7	3GSCR_K24*	JX127179	*Bracteacoccus* sp. 668, U63103 (98 %)	
19				15	3GSG3RE_K15	JX127180	*Bracteacoccus* sp. 668, U63103 (99 %)*	
20				19	3GSCE_K1*	JX127178	*Mychonastes zofingiensis* CCAP 211/14, GU827478 (99 %)/synonym *Chromochloris*	
21				3	3GSG3RE_K21*	JX127176	*Pseudomuriella aurantiaca* strain Bethesda C-1.2.1., X74005 (99 %)	
22		3		2	3GSG1RE_K5*	JX127177	Uncultured Chlorophyta clone DA-01, AB257659 (99 %), *Jenufa* -related	Chlorophyceae

*Representative full-length sequence in phylogenetic analysis.

The composition of the green algal communities in wall areas A and B was apparently different, according to the analysis of clones from environmental DNA (Table 2, Figs. 7-9). Whereas *Apatococcus lobatus* and *Phyllosiphon arisari*-related sequences were highly abundant on wall area A but either absent or rare on wall area B, *P. calcarius*- and *Trebouxia asymmetrica*-related sequences were detected nearly exclusively on wall area B. Only five green algal OTUs were shared between both wall areas, out of which three *Stichococcus*-related OTUs were in fact abundant (green algal OTUs 3, 4 and 8; Table 2).

The composition of the algal community from the basement vault scale samples has little in common with the communities from wall areas A and B (Fig. 7). Just the *Stichococcus minutus* (green algal OTU 4)- and *P. arisari* (green algal OTU 16)-related OTUs were shared by all three sampling sites.

With respect to wall areas A and B, crude cultures revealed only sequences belonging to Trebouxiophyceae, affiliated to green algal OTUs 2, 3 and 4. From the basement vault scale samples, just a single green algal OTU (a *Chromochloris zofingiensis* relative, Chlorophyceae) was recovered in the environmental clone library. In contrast to crude cultures from wall area samples, a high number of otherwise undetected OTUs were retrieved from crude cultures of scale samples (Table 2). Again, *Chromochloris zopfingiensis*-like green alga was abundant in these clone libraries. In addition, *Bracteacoccus*- and *Stichococcus*-related sequences were frequent.

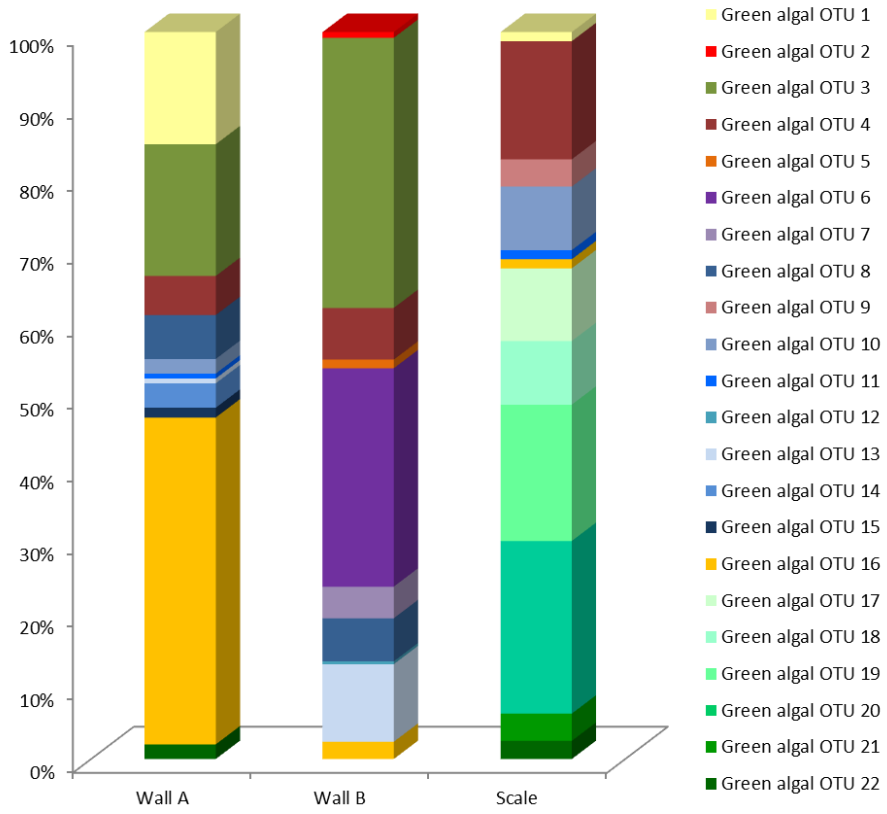

Fig. 7. Relative abundance of green algal operational taxonomic units (OTUs; 98 % cut-off) obtained after analysis of 18S rRNA gene sequences.

In summary, phylogenetic analysis revealed most green algal OTUs belonging to the green algal class of Trebouxiophyceae (16 OTUs; Fig. 8); only 6 OTUs represented Chlorophyceae (Fig. 9). In addition, one member of the Ulvophyceae (*Trentepohlia* sp.) was found in two clones on wall area A (data not shown). Interestingly, on wall areas A and B, Trebouxiophyceae were dominant. Only one OTU, retrieved from environmental DNA cloning on wall area A, a *Jenufa* sp.-related clone, could be assigned to the green algal class of Chlorophyceae. All other chlorophycean clones were retrieved from environmental material and crude cultures of scale samples.

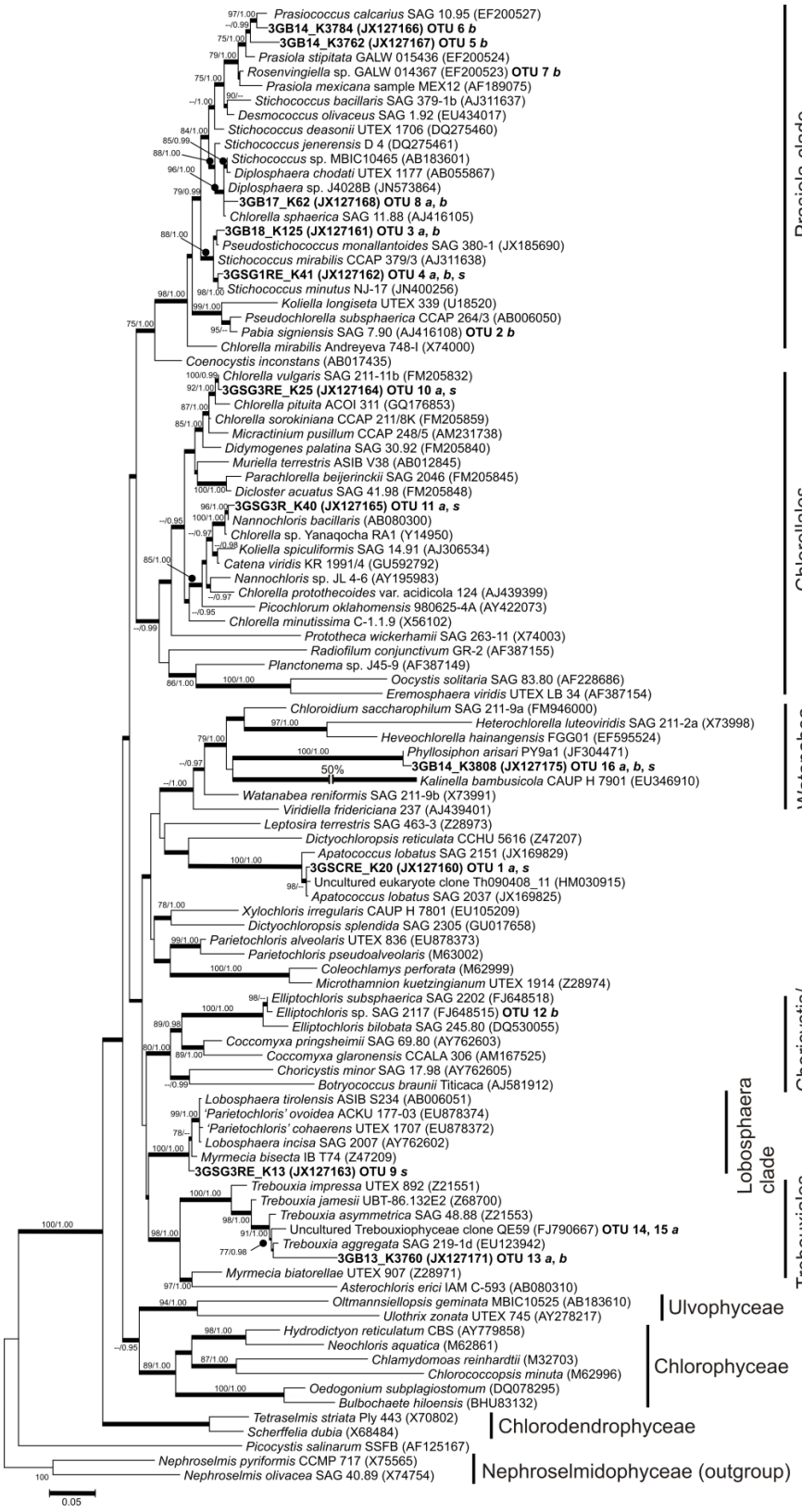

Fig. 8. Phylogenetic tree of Trebouxiophyceae based on 18S rRNA gene sequences. Maximum likelihood method (RAxML) based on 102 taxa and 1798 positions. Thick lines indicate internal nodes that were received in maximum likelihood and Bayesian trees. Support values given as ML (maximum likelihood; > 75 % bootstrap values)/MB (MrBayes; > 0.95 Bayesian posterior probabilities). Sequences in bold represent full 18S rRNA gene sequences of clones; a: wall A; b: wall B, s: scale.

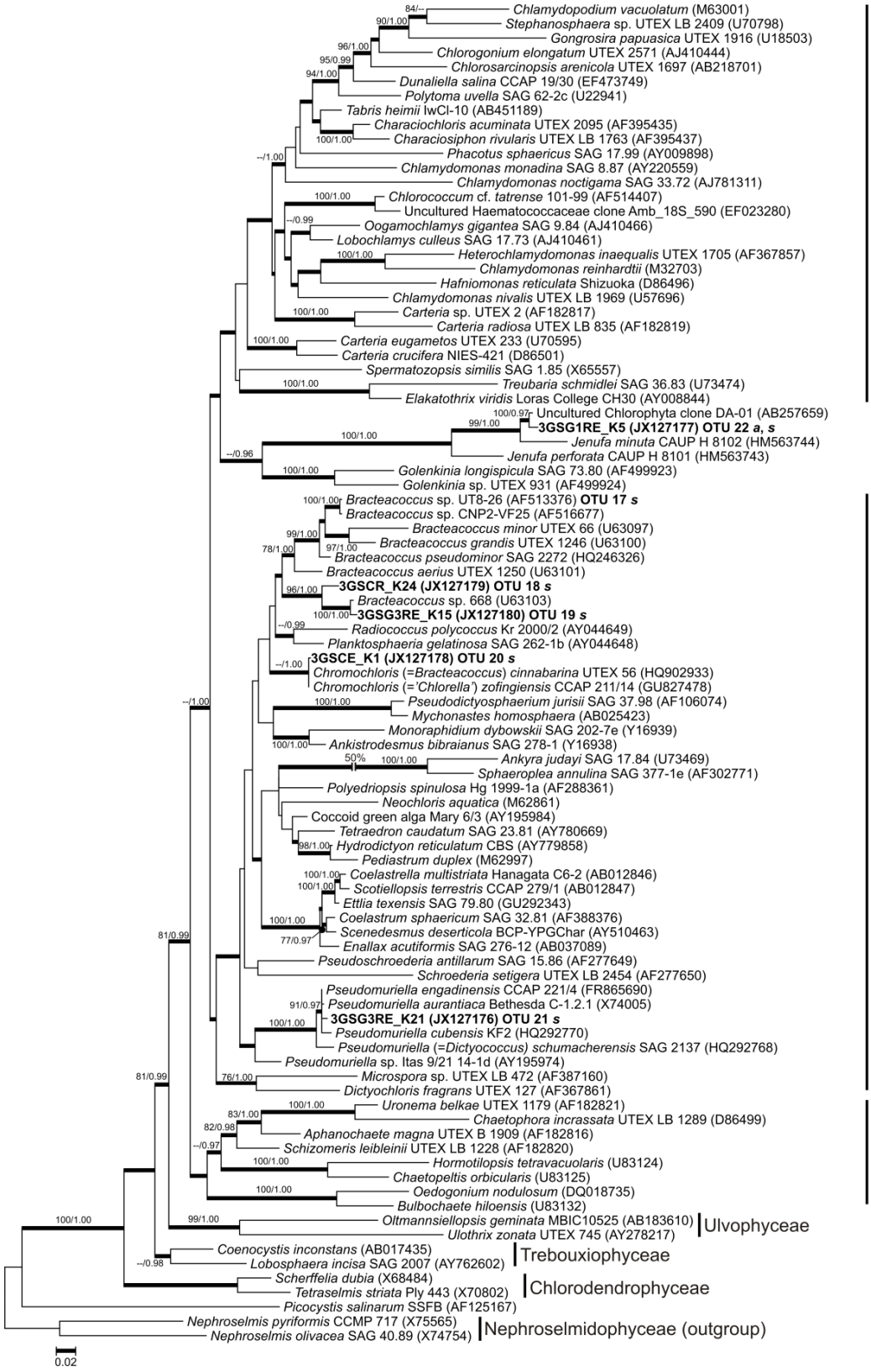

Fig. 9. Phylogenetic tree of Chlorophyceae based on 18S rRNA gene sequences. Maximum likelihood method (RAxML) based on 93 taxa and 1780 positions. Thick lines indicate internal nodes that were received in maximum likelihood and Bayesian trees. Support values given as ML (maximum likelihood; > 75 % bootstrap values)/MB (MrBayes; > 0.95 Bayesian posterior probabilities). Sequences in bold represent full-length sequences of clones; a: wall A; b: wall B, s: scale.

Diatoms

From scale samples developed with diatom specific 18S rRNA gene primers, 39 clones closely related to *Nitzschia amphibia* (AJ867277, 98 % similarity), and 15 clones related to *Phaeodactylum tricornutum* (AJ269501, 96 % similarity) were identified according to BLAST database queries. However, the latter clones were assigned to *Diadesmis* sp. in the ARB phylogenies. In addition, one sequence showed 99 % similarity to *Spumella* sp. strain Mbc 3C (Chrysophyceae).

Distinction among sites

The rRNA gene sequence data were used for statistical analysis to compare community compositions between the different locations. SIMPER analysis documented the dissimilarity between the daylight-exposed wall areas and the endolithic biofilm of scale samples (93.4 % between wall area A and scale samples, and 96.6 % between wall area B and scale samples; ANOSIM significance: $P < 0.01$). In contrast, both the daylight-exposed wall areas showed 76.4 % dissimilarity.

Discussion

The molecular phylogenetic approach in our study revealed clear differences in the composition of green algal and cyanobacterial communities between two wall areas made of apparently similar substrata (sandstone) located at the same building. The wall areas experienced different exposures to sunlight and differed by the presence or absence of gypsum mortar and crusts. The composition of the fungal microbial communities at the same building was already found to differ at small scales in a previous study (Hallmann et al., 2011b). A previous study on different substrata from geographically distant locations revealed the substrate type as most significant factor to determine prokaryotic communities. For eukaryotic communities, the geographic location appeared to be more significant (locations in France and Ireland, Ragon et al., 2012).

Although we used only partial rRNA gene sequences to assess the community compositions, we employed full rRNA gene sequences for robust phylogenetic analyses. 18S rRNA partial gene analysis of the hypervariable regions V2-V4 or just the V4 region are recommended as DNA barcodes for various algal groups, for example dinoflagellates and diatoms (Zimmermann et al., 2011; Ki, 2012; Pawlowski et al., 2012). Similarly, for 16S rRNA genes, the suitability of the hypervariable regions V1-V3 in phylogenetic analyses turned out to be comparable to full-length sequences (Jeraldo et al., 2011). Consequently, partial rRNA gene sequences are still commonly used in microbial biodiversity studies using a culture-independent approach (Santos et al., 2010).

Cyanobacterial communities

There is an obvious difference between the two wall areas concerning the abundance of cyanobacterial clones. Whereas just seven cyanobacterial clones could be retrieved from area B, more than 50 % of the clones recovered from area A represented two OTUs (OTU 3 and 11, c.f. Table 1), that is, *Synechococcus*-like cyanobacteria. The latter two OTUs had their next relatives in BLASTN queries with 98 % and 96 % similarities in *Synechococcus*-like cyanobacteria retrieved from a marble sculpture (JQ404415; China, Beijing, Forbidden City). Phylogenetic analysis (Fig. 5) revealed an uncultured cyanobacterium from gypsum crusts (GQ325750 from Tunisia; Stivaletta et al., 2010) as another next-closest relative. Although our *Synechococcus*-related clones are closely related to each other, generally *Synechoccoccus* strains belong to several deeply branching lineages within the cyanobacteria. This has been shown recently for a number of isolates

mainly obtained from freshwater (Robertson et al., 2001). With respect to our findings, it should be noted that some *Synechococcus* isolates could be retrieved from saline and hypersaline environments, including gypsum crusts (e.g. Oren and Seckbach, 2001; Crispim and Gaylarde, 2005). The predominance of *Synechococcus* in our samples may be also well explained by the presence of a thin gypsum layer on the stone surface (c.f. Fig. 1b). It is generally accepted that cyanobacteria instead of green algae dominate alkali - including hypersaline - environments (e.g. Oren and Seckbach, 2001). Gypsum was one component of the used mortar and may have been dissolved by precipitation (c.f. Hoppert et al., 2010). Typically, the cyanobacteria grow inside the crust, in particular at the boundary layer between crust and stone substratum (Saiz-Jiminez et al., 1990). In the dry and cold Antarctic habitat, hypolithic growth at the contact face between stone and underlying ground has been observed for cyanobacteria (see Cary et al. (2010) for a review). This layer of active growth also defines the fracture plane of the gypsum crust (Hoppert et al., 2010). For *Synechococcus*, in particular, the binding of calcium ions on the negatively charged surface layers of cells has been described, which contributes to dissolution and biogenic weathering of limestone (Schultze-Lam and Beveridge, 1994).

Leptolyngbya (cyanobacterial OTU 2; Table 1) was abundantly recovered under large basement vault scales (c.f. Fig. 1g, h). Here, the fracture face of the scale is composed of clay particles intermixed with gypsum. Moreover, the site is moist and just exposed to dim light for few hours per day. *Leptolyngbya* has been predominant in phototrophic biofilms from hypogean sites (Zammit et al., 2011), but was also detected in diverse endolithic microbial habitats such as travertine (travertine terraces of the Yellowstone National Park), alpine dolomite, and even deep sea basalt and gypsum crusts in Tunisia (Norris and Castenholz, 2006; Horath and Bachofen, 2009; Stivaletta et al., 2010). *Leptolyngbya* OTU 2 is closely related to an uncultured cyanobacterium recovered from a hypolithic biofilm on quartz rock (FJ790618; Wong et al., 2010).

Further cyanobacterial OTUs recovered from clone libraries represented *M. vaginatus* and *Chroococcus* sp. Both species/genera are common cyanobacteria of terrestrial habitats also colonizing dimension stone (e.g. Ortega-Calvo et al., 1991; Cuzman et al., 2010). The cyanobacterial OTUs 4, 5 and 6 from the clone libraries were phylogenetically closely related to *Chroococcidiopsis.* Their close relatives were from terrestrial or hypolithic sites (GQ396895, HM241004, FJ790556, HM224428). The dominance of either *Synechococcus* on wall area A or *Leptolyngbya* and other cyanobacteria in the scale samples may be due to

the different exposure times to direct sunlight, high calcium concentration in gypsum crusts (as compared with scales) or different moisture regimes at these sites.

In fact, filamentous cyanobacteria were only detected on shaded wall area B and in the scale samples. According to a recent study on microbial biofilms on monuments of the Angkor Wat temple complex (Cambodia), filamentous cyanobacteria like *Microcoleus vaginatus* and *Leptolyngbya* appeared to be more prevalent in moist areas as well (internal walls; Gaylarde et al., 2012). However, both filamentous cyanobacteria are also important colonizers of soil crusts and deserts with specific adaptations to desiccation stress like exopolysaccharide sheaths (Garcia-Pichel et al., 2001; Büdel et al., 2009; Pereira et al., 2009).

Eukaryotic algal communities

All eukaryotic algae retrieved in our clone libraries belonged to two classes of green algae, either Trebouxiophyceae or Chlorophyceae. Most OTUs from wall areas A and B were members of Trebouxiophyceae (15 out of 16). Three distinct groups of clones (OTUs 3, 4 and 8), phylogenetically related to *Pseudostichococcus monallantoides* strain SAG 380-1 (*Stichococcus mirabilis* strain CCAP 379/3 with 99 % similarity in BLAST queries), *S. minutus*, strain NJ-17 and *Diplosphaera* sp. strain J4028B (*Stichococcus* sp. strain MBIC10465 with 99 % similarity in BLAST queries), were retrieved in high abundance from both walls (c.f. Table 2 and Fig. 8).

Green algal OTU 5 was recovered only from wall area B and in low clone numbers; it was phylogenetically affiliated to *P. calcarius* (*Stichococcus* sp. strain MBIC10465, with 98 % similarity in BLAST queries). Another *P. calcarius*-related sequence, OTU 6, was also found only at wall area B, but with high clone numbers. For OTU 6, both phylogenetic analyses and BLAST queries concurred on the species same identification, the cell package forming *P. calcarius* (c.f. Table 2, Figs. 3a, 8). The species has been described as a widely distributed subaerial alga on moist soil, calcareous rock and stone walls (Ettl and Gärtner, 1995), including the Antarctic region (Belcher, 1969; Broady, 1983). There, *P. calcarius* was observed to be dominant in epilithic communities at high-salinity sites where irradiation is infrequent or moisture is available (Broady, 1996).

Obviously, the '*Stichococcus*' morphotypes within the *Prasiola* clade are phylogenetically diverse and also differ with respect to their abundance in specific sites. In scale samples, the *S. minutus*-related clones could be retrieved just from crude cultures.

No environmental clone from scale samples, though, was related to *Stichococcus*, which accounts for a minor 'contamination' of the site by this clone.

Surprisingly, *A. lobatus*-related clones could not be retrieved from shaded wall area B. On wall area B, the highly abundant *Prasiococcus* (being absent on wall area A) appears to be better adapted to this site. *Apatococcus*, like *Stichococcus*, is known as a genus of cosmopolitan algae. Cell packages of *A. lobatus* were described from tree bark, wood, walls, rocks (Ettl and Gärtner, 1995) and man-made substrata (Rindi et al., 2010). The sequences revealed in this study were closely related to *A. lobatus* strain SAG 2037. The resistance of *Apatococcus* against air pollution in towns and xeric conditions is well known (Barkman, 1969). In this particular case on wall area B, *Apatococcus*, although exhibiting wide ecological amplitude, might be less competitive. As *Apatococcus* is mixotroph (Gustavs, 2010) and is able to grow under shaded conditions, eventually lack of organic substrates on this site may be a reason for the absence of *A. lobatus*-related clones.

Clones closely affiliated to the rather uncommon green alga *P. arisari* Kühn could be retrieved from wall area A in high numbers (six sequences from area B, one sequence from scale samples). The green alga *P. arisari* has been described as a plant parasitic alga penetrating *Arisarum* leaves in coastal Mediterranean and in tropical climates, but was also described for temperate climates (Aboal and Werner, 2011). Due to this fact, it can be assumed that the reference sequence is a misidentification. This reference sequence, in fact, seems to be assigned to *Lobosphaeropsis pyrenoidosa* Reisigl (T. Darienko, personal communication). *Lobosphaeropsis pyrenoidosa* was discovered in soil (Ettl and Gärtner, 1995), on tree bark (Freystein et al., 2008) and on stone (T. Darienko, personal communication).

From wall area A, only one chlorophycean alga related to *Jenufa minuta* was retrieved. Isolates were obtained from tree bark in Singapur (Němcová et al., 2011) and from soil in Germany (Hodač et al., 2012), and detected in clone libraries from endolithic samples in the Alps, Switzerland (Horath and Bachofen, 2009). All six chlorophycean OTUs detected in this study were retrieved from crude cultures of scale samples. Interestingly, enrichment cultures of samples from other wall areas did not result in an enrichment of Chlorophyceae, which accounts for a 'seed bank' of chlorophycean algae particularly in scales. Except *Jenufa*, these Chlorophyceae belong to the order Sphaeropleales, which includes vegetatively non-motile unicellular or colonial taxa (Lewis and McCourt, 2004).

SEM of scales revealed the dominance of one coccoid morphotype (Fig. 2b), along with cyanobacteria and diatoms, from the basement vault scale sample. This morphotype was assigned to an OTU related to *C. zofingiensis* (GU827478, formerly *Chlorella*, *Muriella*, '*Myconastes*'; Hindák, 1982; Ettl and Gärtner, 1995; Krienitz et al., 2011; Fucíková and Lewis, 2012). The same OTU was also abundant in environmental samples from scales. The aerophytic alga has been found on rather sandy soil and moist substrata. This implies that *Chromochloris* is adapted to rather moist terrestrial environments (Ettl and Gärtner, 1995).

As the other chlorophycean OTUs could only be retrieved from crude cultures, they must be present just in minor proportions in the original sample. These clones are, like *Chromochloris*, rather soil algae than algae normally found on hard rock substrata and on wall areas A and B. This feature is also pointed out by SIMPER analysis: wall areas A and B are clearly distinct from the scale samples, in particular caused by presence/absence of Chlorophyceae.

The diatoms observed in the scale samples are cosmopolitan species, not necessarily restricted to terrestrial habitats. *Diadesmis contenta* is known, for example, for cave systems in Austria and Czech Republic (Schagerl, 1991; Poulíčková and Hašler, 2007) and was also found in lichen thalli (Lakatos et al., 2004). *Achnanthidium minutissimum* was retrieved, for example from aquatic systems (Potapova and Hamilton, 2007) and caves (Poulíčková and Hašler, 2007). Interestingly, one clone related to the flagellate *Spumella* sp. was retrieved in the scale samples. Chrysophyceaen algae are well known from freshwater and soil habitats (Boenigk et al., 2005), but could be also retrieved from moss (Škaloud, 2009).

Diversity patterns of the phototrophic community and possible biodeterioration mechanisms

It is reasonable to assume that algal communities found in the dark and moist environment of a basement vault differ from communities exposed to sunlight and, hence, to desiccation stress. However, it may not be expected that algal communities on two walls with (seemingly) similar environmental conditions are clearly distinct from each other.

Although undoubted reasons for these distinctions have yet to be elucidated, two differences between the wall areas are obvious: (1) different sun exposure time per day attended by water availability and (2) wall joints filled with gypsum mortar on wall area A in contrast to open wall joints on wall area B, resulting in the formation of thin gypsum

crusts on wall area A. This accounts for clearly distinct ecological adaptations of microalgae on either wall area A or wall area B. It should be taken into account that in addition to the crucial criterion of the available moisture, also the chemical properties of the substratum substantially influence the settlement of specific algae (Darienko and Hoffmann, 2003; Rindi, 2007).

However, crude culturing leads to enrichment of other algal genera that may be well present in low numbers in the environment, but readily capable of multiplying under appropriate conditions. Obviously, these 'seed banks' differ between the various sampled sites. Although various Chlorophyceae are present in basement vault scales, the same genera appear to be nearly completely absent on walls.

Knowledge on the diversity, ecophysiology and dispersal strategies of these algae are still in its infancy (Karsten et al., 2005; Rindi, 2007). The different ecological adaptations of, for example, *Lobosphaeropsis pyrenoidosa* (*P. arisari*) or *P. calcarius* being dominant on either wall area A or B are largely unknown. Generally, algae exhibit a variety of adaptations to the terrestrial habitat (Häubner et al., 2006). Dry periods (in summer with low precipitation or due to frost drought) require adaptations to desiccation. These adaptations are manifested by certain cytological features such as thickened cell walls (*Klebsormidium*, Holzinger et al., 2011; Karsten and Holzinger, 2012; *Zygogonium*, Hoppert et al., 2004) and mucilaginous sheaths (*Coccomyxa*, Karsten et al., 2005). These structures may retain water for a certain time period and help to withstand high osmotic stress during desiccation. The accumulation of exopolysaccharides (EPS) as a protection against desiccation has already been reported (Shepherd and Beilby, 1999). Equally important are mechanisms protecting against high radiation, such as the accumulation of carotenoids and the formation of MAAs (mycosporine-like amino acids; Karsten et al., 2007b). Additionally, the presence of polyols, for example ribitol, which were considered as effective stress metabolites, was demonstrated in representative aeroterrestrial algae (Gustavs et al., 2011). Moreover, resting stages such as spores persist for years (Karsten et al., 2005).

In a recent study, different wall areas of the castle Gleichen were mapped, and distinct weathering patterns like formation of crusts or flakes and salt efflorescences were reported for wall area A (Stück et al., 2011). In our study, no direct evidence for biodeterioration, such as traces of actively penetrating endolithic organisms or mineral dissolution around single cells by microalgae, could be found, although it must be expected that the mass development of cyanobacterial and algal layers will destabilize pores and clefts just by

mechanical forces (Warscheid et al., 1991; Crispim and Gaylarde, 2005). The secretion of organic acids at least by some organisms must be expected. It is obvious that the algae and cyanobacteria use pre-existing fracture planes under crusts or scales (these fracture planes were also observed without any apparent colonization), but may then accelerate detachment of these features. Just because of the high number of individuals (as implied by clone library data), key players of colonization and therefore putative agents of biogenic weathering could be clearly defined: In case of wall area A, the abundant cyanobacterium *Synechococcus* (-like), but also *Phyllosiphon* (*Lobosphaeropsis pyrenoidosa*) and *Stichococcus*/*Pseudostichococcus*, must be taken into account. On wall area B, besides *Stichococcus*, *Prasiococcus* was highly abundant. Finally, the basement scale sample was dominated by *Chromochloris zopfingiensis* and *Leptolyngbya*. These dominating organisms were also accompanied by a set of distinct other species, except from a few generalists (in our case, just *Stichococcus*). It has to be expected that these organisms also exert different mechanisms of biogenic weathering on the material surface. Thus, also effects of biogenic weathering regimes may differ on a very small scale.

Factors that influence the dominance of specific phylotypes were obviously irradiance, moisture and presence or absence of gypsum crusts. However, a more detailed analysis of the relevance of these determinants will require further quantification of these physical and chemical parameters of the substratum and long-term measurements of microclimatic conditions.

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Chapter 2:
Cryptogam covers on sepulchral monuments and re-colonization of a marble surface after cleaning

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Analyzed data: CH.
Cleaning procedures of the sculpture: WW.
Performed scanning electron microscopy: DHR, CH.
Wrote the paper: CH, MH.
Conceived and designed the experiments: MH, CH, WW.

Abstract

Re-colonization of freshly cleaned surfaces by aeroterrestrial microbial communities is up to now poorly understood. Here, we present a comparative study addressing the composition of algal and fungal communities on a marble sculpture, based upon the analysis of 18S rRNA gene clone libraries from environmental samples. The samples were taken from a blackish and greenish biofilm cover before surface cleaning and 1 year after cleaning treatment, when traces of re-colonization became visible to the naked eye. The composition of the fungal community indicated clear differences between the old grown biofilm and the treated surface. While the former was dominated by the ascomycetes *Rhinocladiella*, *Glyphium* and Capnodiales, the black yeast *Sarcinomyces* was clearly dominant 1 year after cleaning, but could not be retrieved from the old grown black biofilm. The green algal community was dominated by different phylotypes of the lichen algae *Trebouxia*, as well as the cosmopolitan green algae *Apatococcus* and *Stichococcus*. No essential differences in the green algal community before and after cleaning could be observed.

Keywords: environmental samples, fungi, green algae, lichen, 18S rRNA gene.

Introduction

Open-air sculptures and historic buildings exposed to weather and pollution are deteriorated by physical and chemical factors, as well as by microbial contamination (Saiz-Jimenez, 1995; Zanardini et al., 2000; Polo et al., 2010).

Microbial communities on stone surfaces are composed of bacteria including cyanobacteria, fungi, algae and lichens (Warscheid and Braams, 2000; McNamara and Mitchell, 2005). Phototrophs, like cyanobacteria and algae, are primary colonizers (Grant, 1982) and also provide nutrients for heterotrophic microorganisms, e.g. fungi (Gaylarde et al., 2012). All these microorganisms may be involved in biodeterioration, e.g. biopitting by penetration of fungi in the material surface but also as staining caused by algae and black pigmented fungi (Sterflinger and Krumbein, 1997; McNamara et al., 2006; Polo et al., 2010 and references therein). A multitude of studies documented the deterioration of marble monuments and building facades by weathering, pollution and microbial communities (Gromov, 1963; Lamenti et al., 2000; Gorbushina et al., 2002; Moropoulou et al., 2002; Cappitelli et al., 2007; Weber et al., 2007; Sert and Sterflinger, 2010; Hallmann et al., 2011a).

Different cleaning methods and conservation procedures were developed, based on chemical compounds (Young and Urquhart, 1998; Moropoulou et al., 2002; Polo et al., 2010), excimer laser cleaning (Maravelaki-Kalaitzaki et al., 1999), protective synthetic resins (Cappitelli et al., 2007) or application of biomimetic apatite (Yang et al., 2012). Even biological treatments with sulfate reducing bacteria for black crust removal were established (Cappitelli et al., 2006; Konkol et al., 2008).

So far only few studies were devoted to comparative analysis concerning re-colonization by microorganisms after cleaning procedures. Lamenti et al. (2000) observed the re-colonization of marble statues by phototrophic biofilms for 6 years after restoration by culturing method. Polo et al. (2010) analyzed microbial communities by denaturing gradient gel electrophoresis (DGGE) before and immediately after biological and chemical remediation of oolithic limestone sculptures. Lan et al. (2010) stated obvious differences between the eukaryotic community of an old and a newly developed biofilm on sandstone of a Temple building of Angkor Thom in Cambodia, while the bacterial communities of both biofilms were almost similar.

In our study, an ‘‘old grown’’ blackish and greenish biofilm (surface left untreated tens of years) and a newly developed biofilm on the surface of a sepulchral marble

sculpture 1 year after cleaning were analyzed and compared by environmental cloning and sequencing. The focus was on green algal and fungal communities, to identify the emerging colonizers in an early period of (re-) colonization.

Materials and methods

Sampling

The sampling site was a marble sculpture on the historical Bartholomäus cemetery in Göttingen, Lower Saxony, Germany (51°32′27.49″N, 9°55′54.52″E). The sculpture was created in 1802 by the sculptor Johann Christian Ruhl (1764-1842) and was placed on the grave of Carl von Hahn. The grave was situated under a tree and near a main street.

The sculpture, made of Tuscan Carrara marble, was covered with a blackish and greenish biofilm dominated by fungi and green algae. At some spots, also crustose and foliose lichens were abundant (cf. Figs. 1a, b, 2a). The grave was restored in 2009/2010, including a surface cleaning of the sculpture. The cleaning was performed as follows: the cryptogam cover was removed with soft brushes and wooden tools; then, the surface was cleaned with a vapor stem cleaner. Since dark stains could be not removed by this procedure, the surface was treated several times with a diluted hydrogen peroxide solution. A resulting foam layer on the surface was removed by rinsing with water. The surface was then impregnated with a thin layer of calcium silicate. A small area situated on the back of the sculpture was left untreated. All samples were collected in April 2011, 1 year after cleaning and restoration. Samples of approx. 100 µl dry volume were taken from the cleaned white marble monument (‘‘cleaned surface’’, sample A) and from the small area covered with the old grown black biofilm (‘‘uncleaned surface’’, sample B) which had been left untreated during cleaning. The samples were scraped off with a sterile scalpel and collected in sterile 1.5 ml reaction tubes.

DNA extraction

Genomic DNA was extracted from collected environmental biofilm samples. For the cell disruption by shaking in a Mini-BeadBeater (Biospec Products, Bartlesville, OK, USA) equivalent amounts of acid washed glass beads (120-200 µm and 425-600 µm in diameter; Sigma-Aldrich, ST. Louis, MO, USA) were added to 2 ml reaction tubes containing the samples. The samples were treated in the bead beater for 30 s at 5.000 rpm.

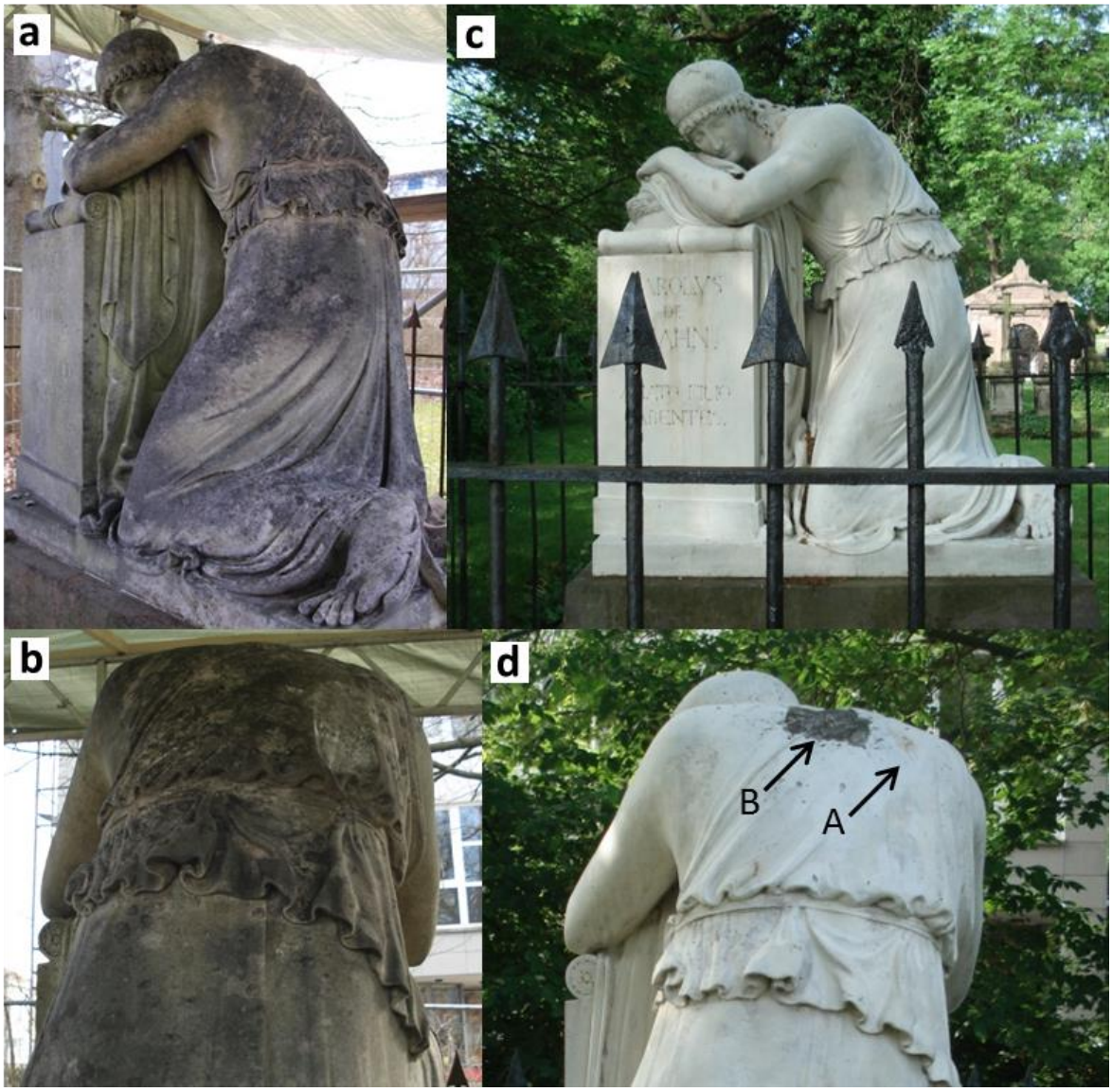

Fig. 1. Sampling site on sepulchral monument. (a) Monument covered with blackish and greenish biofilm. (b) Back view of the untreated monument. (c) Monument 1 year after cleaning treatment. (d) Back view after cleaning. The arrows mark the sites where samples A and B were taken (A cleaned surface, B uncleaned surface).

DNA was extracted with the Invisorb Spin Plant Mini Kit (STRATEC Molecular, Berlin, Germany), following the manufacturer's instructions. Extraction results were checked on a 1 % (w/v) agarose gel. Isolated DNA was stored at -20 °C until further processing.

Polymerase chain reaction amplification

For isolated biofilm DNA the polymerase chain reaction (PCR) amplification was performed with two primer combinations for 18S rRNA gene, using eukaryotic standard primers 20F (5' GTAGTCATATGCTTGTCTC 3') and

18L (5' CACCTACGGAAACCTTGTTACGACTT 3'); Hamby et al., 1988). In a second approach, the samples were first amplified with 20F/18L and in a second round (semi-nested PCR) with 20F and the green algal-specific primer CH1750R (5' CTTCCTCTAGRTGGGAGG 3'; Hallmann et al., 2013). About 30 ng of the extracted DNA was used as template. The amplification reaction mixture (25 µl) contained each dNTP at a concentration of 0.1 mM, 5 µl of 10 x reaction buffer, 2 mM $MgCl_2$, each primer at a concentration of 0.2 µM, 2 U of Taq DNA polymerase (Bioline, Luckenwalde, Germany) and 4 % (v/v) dimethyl sulfoxide (DMSO)-solution. PCR was performed in a thermocycler TProfessional Basic (Biometra, Göttingen, Germany) using the following program for the primer set 20F/18L: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 3 min and final extension at 72 °C for 10 min. For the semi-nested PCR with the primer set 20F/CH1750R, the first PCR product of 20F/18L was diluted 1:25 and used as template; then the following program was used: initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 3 min, and final extension at 72 °C for 10 min. The PCR products were purified using the Invisorb Spin PCRapid Kit (STRATEC Molecular). Aliquots of 2 µl of purified amplicons were analyzed by electrophoresis on a 1 % (w/v) agarose gel.

18S rRNA gene cloning and sequencing

Cloning was carried out with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) with TOP 10 chemically competent one Shot *Escherichia coli* cells (Invitrogen), as supplied by the manufacturer. All eukaryotic clones were sequenced with the 18S rRNA gene standard sequencing primer 895R (5' AAATCCAAGAATTTCACCTC 3') resulting in partial sequences including the hypervariable regions V2-V4 (Hodač et al., 2012). Sequencing reactions were performed by Macrogen Inc. (Seoul, South Korea).

Phylogenetic analysis

The sequences were manually corrected using the sequence analysis program SeqAssem (Hepperle, 2004). Sequences shorter than 400 bp were excluded from further analysis. These sequences were compared with similar sequences of reference organisms by performing a BLASTN search at NCBI (Altschul et al., 1990; http://www.ncbi.nlm.nih.gov/). Next relative sequences were imported into the ARB program (Ludwig et al., 2004; http://www.arb-home.de). In addition, sequences provided by SAG Culture Collection of

Algae (University of Göttingen) were included in the comparisons. To determine the first phylogenetic affiliation the partial sequences were aligned with the homologous eukaryotic 18S rRNA gene sequences using the automatic alignment tool of the ARB program package. Potential chimeras were checked by Bellerophon (Huber et al., 2004). In addition, the first and the last 300 bp of putative chimeras were compared with similar rRNA gene sequences in NCBI. Chimeras were excluded from the dataset.

Rarefaction curves and operational taxonomic units (OTUs) were calculated with MOTHUR (Schloss et al., 2009). OTUs were defined on ≥ 98 % sequence similarity for 18S rRNA genes (Ragon et al., 2012). One sequence of each OTU with respect to green algae was selected and sequenced completely with 18S standard sequencing primers: 34F, 370R, 891F, 1122F, 1122R, 1422F. Representative sequences were deposited in GenBank under the following accession numbers: JX391005 - JX391026. The alignments for phylogenetic analysis were performed using MAFFT version 6 (Katoh and Toh, 2008); small corrections were done by eye. The phylogenetic tree was constructed with full-length sequences using the RAxML search algorithm for maximum likelihood (ML; Stamatakis et al., 2008), using the GTR+Γ+I model with 100 replicates. The confidence of the tree topologies was tested by bootstrap analysis implemented in RAxML (100 replicates) and by Bayesian posterior probabilities (MB) using MrBayes 3.2 (Huelsenbeck and Ronquist, 2001). Two parallel Markov chain Monte Carlo (MCMC) runs for two million generations each with one cold and three heated chains were conducted using the GTR+Γ+I model, with trees sampled every 100 generations.

Light and scanning electron microscopy

Light microscopic observations were performed using an Olympus BX60 microscope (Tokyo, Japan) with Nomarski DIC optics with a ColorView III camera (Soft Imaging Systems, Münster, Germany) attached and micrographs were processed using the Cell^D image software (Soft Imaging Systems). For scanning electron microscopy (SEM), samples were fixed immediately after sampling in 2 % w/v glutardialdehyde (EM grade, Sigma-Aldrich, Deisenhofen, Germany) and stored at 4 °C until further processing. Samples were dehydrated in an ascending ethanol series (15 % to 99 %), mounted on SEM sample holders and sputtered with Au-Pd (13.9 nm for 120 s). Samples were visualized in a SEM LEO 1530 Gemini (Zeiss, Oberkochen, Germany) combined with an INCA X-ACT EDX.

Results

Before surface cleaning, the marble sculpture exhibited obvious colonization by cryptogams, including fungi, algae and lichens (Fig. 1a, b). Surface cleaning was done mechanically with water, diluted hydrogen peroxide solution and a soft brush; if necessary, also mild detergent was used. After cleaning, the white marble appeared to be completely free of surface staining or colonization to the naked eye, except from a rectangular 5 x 10 cm area, which remained untreated. During cleaning, care was taken that neither water nor chemicals came in contact to the uncleaned area. One year after cleaning treatment (Fig. 1c, d) the monument appeared still white but some blackish spots, visible to the naked eye, already reappeared. On the sampling area B thalli of the foliose lichen *Physcia* sp. were obvious (Fig. 2a). SEM of a lichen thallus revealed reproductive structures like soredia (Fig. 2b, b_1). Light microscopy showed supposedly pre-lichenized *Trebouxia* cells surrounded by fungal hyphae (Fig. 2c). Lichen thalli were attached to the substrate by rhizines. These rootlike fungal structures were firmly associated with mineral particles detached from the surface of the sculpture (SEM, Fig. 3a-d). SEM also revealed the presence of a plant pathogenic fungus on the lichen thallus. A chasmothecium (fruiting body) of a powdery mildew fungus (family Erysiphaceae) was anchored by appendices on the substratum (Fig. 3e, f).

Sequencing of 18S rRNA genes aiming at detection of eukaryotic algae and fungi was performed for environmental samples. In total 222 18S rRNA gene sequences were retrieved, 112 sequences from sample A (cleaned surface) and 110 sequences from sample B (uncleaned surface). Overall, 22 OTUs were received on the 98 % sequence similarity level. Altogether 17 OTUs were retrieved from sample A and 14 OTUs from sample B. Rarefaction curves (Fig. 4) calculated for both samples showed the clone library coverage for algal and fungal sequences. The rarefaction curve from sample B reached almost a plateau. This was not the case for sample A, though also nearly full coverage of OTUs was reached. With respect to green algae the samples indicated few differences (Fig. 5). Altogether ten green algal OTUs were retrieved, nine OTUs (69 clones) from sample A and seven OTUs (71 clones) from sample B. Phylogenetic analysis (Fig. 6) revealed that all sequences belong to the green algal class Trebouxiophyceae.

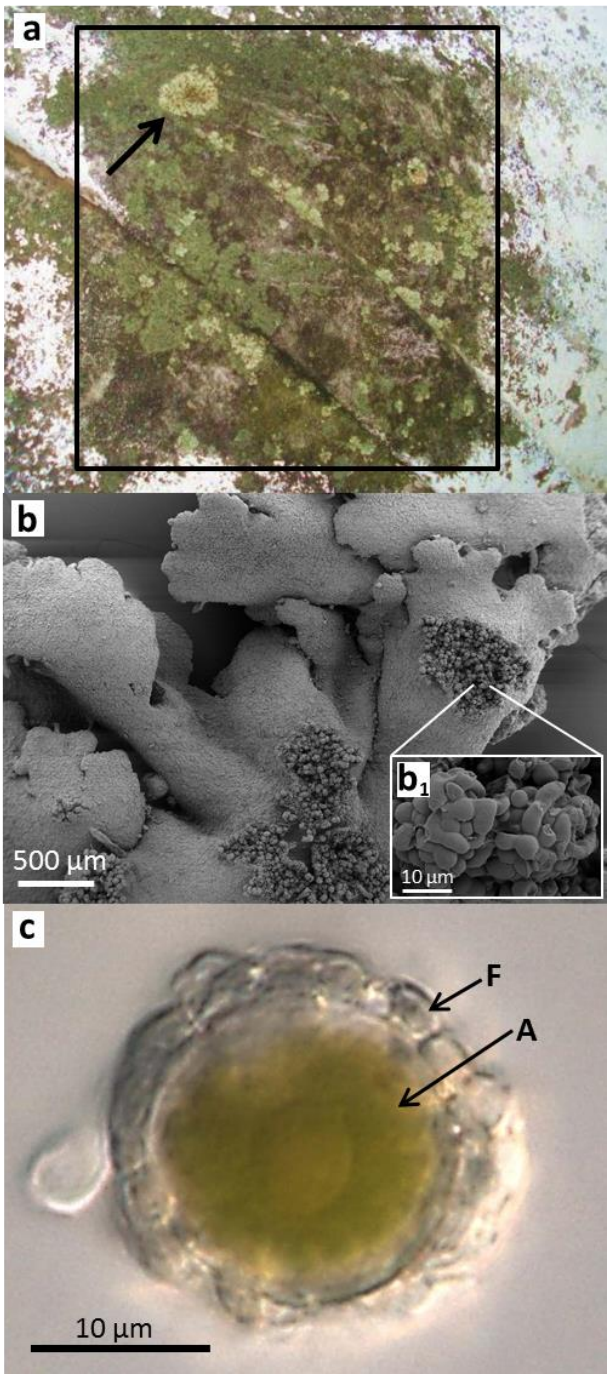

Fig. 2. Lichen thalli on old grown biofilm (sampling site B). (a) Overview image of sampling site B, with lichen thallus of *Physcia* sp. (black arrow). (b) Scanning electron micrograph of a lichen thallus with several soralia, inset (b_1) close-up view of soredia in a soralium. (c) Light micrograph of a lichenized *Trebouxia* cell (F fungal hyphae, A algal cell).

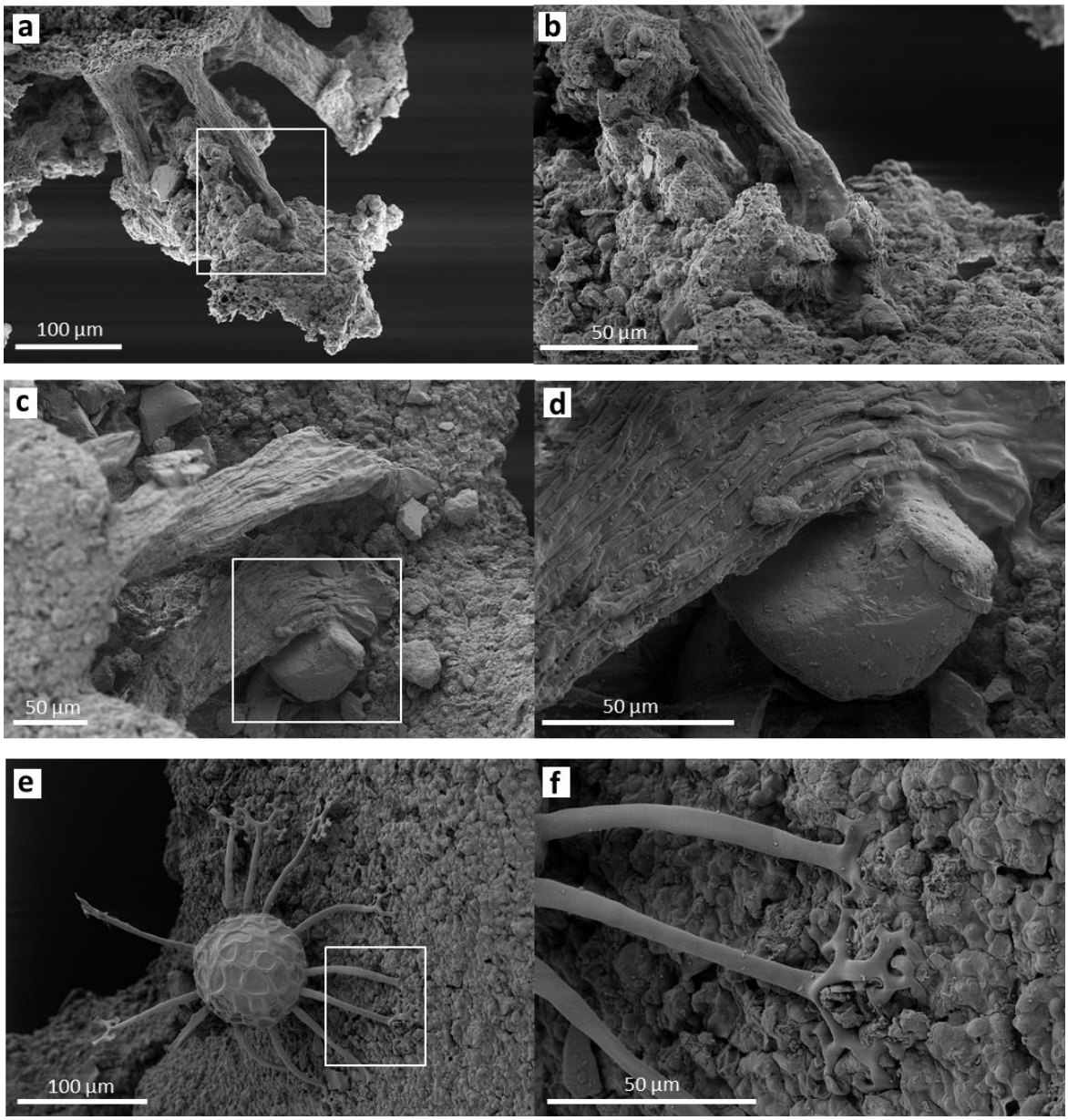

Fig. 3. Features of lichen thalli taken from old grown biofilm visualized by scanning electron microscopy. (a), (c) Overview of lichen-fungi hyphae associated with mineral particles, (b), (d) in detail. (e), (f) Chasmothecium of an Erysiphaceae representative attached on a lichen thallus, (f) detail of appendices of the chasmothecium anchored to the substratum.

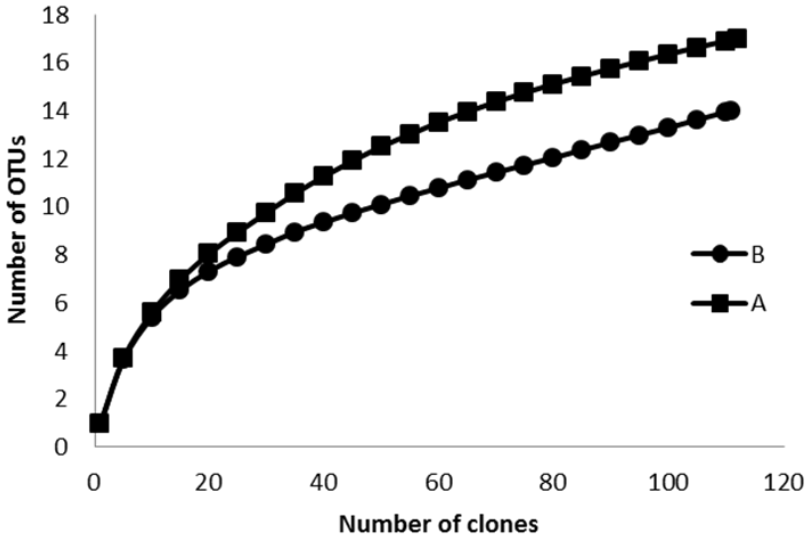

Fig. 4. Rarefaction curves determined for 18S rRNA gene clone libraries. The OTUs include green algal and fungal sequences from samples taken after cleaning treatment (A) and from the uncleaned surface (B).

The green algal composition indicated a high similarity between both samples, six OTUs were shared by both (Table 1), one OTU were found only in sample B and three OTUs in sample A. In both sampling areas the *Trebouxia*-related OTU 5 showed a clear dominance, with 27 clones from sample A and 34 clones from sample B (Table 1; Fig. 6). Furthermore, four OTUs could be assigned to other *Trebouxia*-related OTUs, with high clone numbers for OTU 4 and OTU 6 in both sampling areas. *Stichococcus*- and *Diplosphaera*-related sequences were more abundant in sample A than in sample B, *Desmococcus*- (OTU 1) and *Apatococcus*-related (OTU 10) sequences were detected exclusively in sample A. *Apatococcus* sp.-related sequences could be separated in two distinct OTUs (Fig. 6), phylogenetically affiliated to *Apatococcus lobatus* SAG 2151 (OTU 10, exclusively in sample A, see above) and *Apatococcus lobatus* SAG 2037 (OTU 9), with respectively four clones in samples A and B.

With regard to fungi, both sampling areas differ markedly (Fig. 7). Altogether 12 fungal OTUs were retrieved, eight OTUs (43 clones) from sample A and seven OTUs (39 clones) from sample B. Just three OTUs were shared by both sampling sites (Table 2); next relatives according to BLASTN analysis were members of the family Teratosphaeriaceae (OTU 11), *Rhinocladiella* sp. (OTU 19) and *Glyphium elatum* (OTU 20).

The most abundant OTU in sample B was *Rhinocladiella* sp. (OTU 19, 22 clones), with 99 % sequence similarity to the reference as revealed by BLASTN, while only two

Table 1. Distribution of green algal OTUs in the samples of the cleaned (A) and uncleaned (B) surface.

	No. of clones				
OTU 98 %	A	B	Representative clone	Acc. No.	Closest relative (% similarity)
1	1		FGSwan_K6/(A)	JX391007	*Desmococcus olivaceus* SAG 1.92, EU434017 (99 %)*
2	4	1	FGSsan_K35/(B)*	JX391005	Trebouxiophyte sp. UR55/3, AY762606 (99 %), *Stichococcus* sp.
3	5	1	FGSwa_K37/(A)	JX391006	*Diplosphaera* sp. W1268, JN573889 (99 %)*
4	6	16	FGSsan_K9/(B)*	JX391008	Uncultured marine eukaryote clone NA2_1H8, EF526889 (99 %), *Trebouxia* sp.
5	27	34	FGSwan_K31/(A)*	JX391009	Uncultured marine eukaryote clone NA2_1H8, EF526889 (99 %), *Trebouxia* sp.
6	19	14	FGSwan_K33/(A)*	JX391010	*Trebouxia impressa* UTEX 892, Z21551 (99 %)
7	2		FGSwa_K17/(A)	JX391012	Uncultured eukaryote clone Th090408_47, HM030919 (99 %)*, *Trebouxia* sp.
8		1	FGSsa _K43/(B)	JX391011	Uncultured Trebouxiophyceae clone QE44, FJ790663 (98 %)*, *Trebouxia* sp.
9	4	4	FGSwa_K32/(A)*	JX391013	Uncultured eukaryote clone Th090408_11, HM030915 (99 %), *Apatococcus* sp.
10	1		FGSwa_K16/(A)*	JX391014	Uncultured eukaryote clone Th090408_11, HM030915 (99 %), *Apatococcus* sp.

*Representative full-length sequence in phylogenetic analysis.

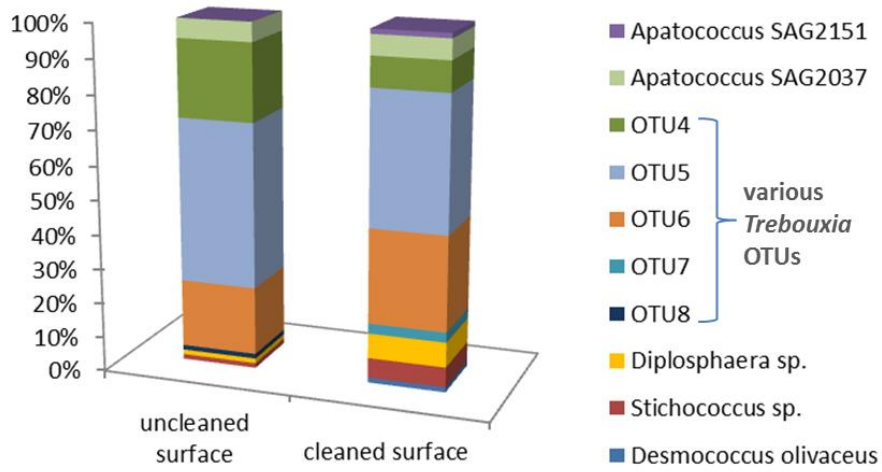

Fig. 5. Distribution of green algal OTUs (98 % cutoff) revealed few differences between the uncleaned surface and the surface after cleaning.

clones were discovered in the sample A. OTUs exclusively found in sample B were related to *Bulgaria inquinans* (OTU 13), a Capnodiales family member (OTU 15), *Caloplaca demissa* lichen fungus (OTU 16) and a Davidiellaceae family member (OTU 17). The most abundant OTU in sample A 1 year after cleaning treatment was *Sarcinomyces* sp.-related OTU (OTU 14, 31 clones), with 99 % similarity to the reference. Furthermore, exclusively found in this sample were OTUs related to *Cladosporium bruhnei* (OTU 12), *Exophiala aquamarina* (OTU 18), the uncultured Banisveld eukaryote clone (100 % sequence similarity)/*Rhodotorula aurantiaca* JCM 3771 (AB030354) with 99 % sequence similarity (OTU 21) and *Cryptococcus albidus* (OTU 22).

Fig. 6. Phylogenetic tree of Trebouxiophyceae based on 18S rRNA gene sequences. Maximum likelihood method (RAxML) based on 89 taxa and 1.801 positions. Thick lines indicate internal nodes that were received in maximum likelihood and Bayesian trees. Support values given as ML (50 % bootstrap values)/MB (0.95 Bayesian posterior probabilities). Sequences in bold represent full 18S rRNA gene sequences of clones; A cleaned surface, B uncleaned surface.

Table 2. Distribution of fungal OTUs in the samples of the cleaned (A) and uncleaned (B) surface.

	No. of clones				
OTU 98 %	**A**	**B**	**Representative clone**	**Acc. No.**	**Closest relative (% similarity)**
11	3	1	FGSwe_K21/(A)	JX391015	*Teratosphaeriaceae* sp. strain CCFEE 5508, GU250358 (99 %)
12	1		FGSwe_K48/(A)	JX391016	*Cladosporium bruhnei* strain CPC 5101, AY251096 (99 %)
13		1	FGSsan_K4(/B)	JX391019	*Bulgaria inquinans* isolate 93, EU107259 (98 %)
14	31		FGSwe_K18/(A)	JX391018	*Sarcinomyces* sp. isolate MA 4760, AJ972809 (99 %)
15		8	FGSse_K24/(B)	JX391017	Capnodiales sp. strain CCFEE 5502, GU250357 (100 %)
16		1	FGSse_K7/(B)	JX391024	*Caloplaca demissa* , AF515609 (98 %)
17		1	FGSse_K17/(B)	JX391020	Davidiellaceae sp. strain CCFEE 5414, GU250343 (97 %)
18	1		FGSwe_K3/(A)	JX391021	*Exophiala aquamarina* strain CBS 119918, JN856012 (98 %)
19	2	22	FGSse_K11/(B)	JX391022	Rhinocladiella sp. Isolate MA 4765, AJ972862 (99 %)
20	1	5	FGSse_K4/(B)	JX391023	*Glyphium elatum* strain CBS 268.34, AF346419 (99 %)
21	3		FGSwe_K23/(A)	JX391025	Uncult. Banisveld euk. clone P1-3m6, EU091832 (100 %), *Rhodotorula aurantiaca* (99 %)
22	1		FGSwe_K28(A)	JX391026	*Cryptococcus albidus* strain WY-1, HQ231895 (99 %)

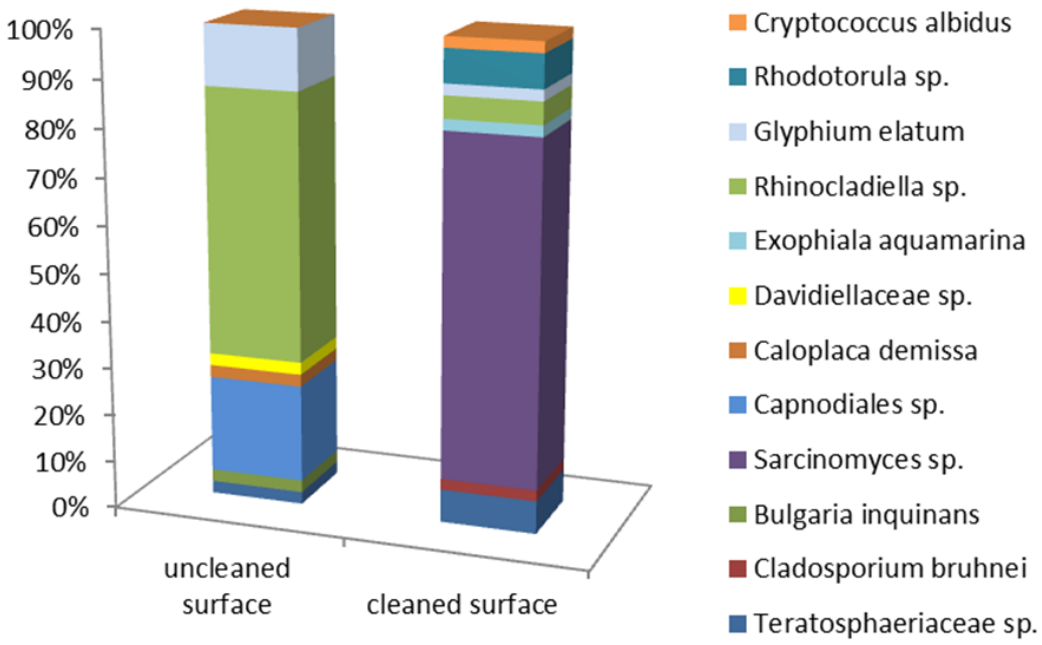

Fig. 7. Distribution of fungal OTUs (98 % cutoff) revealed clear differences between the uncleaned surface and the surface after cleaning.

Discussion

Colonization of monuments, including marble sculptures, has been already documented in various studies (e.g. Albertano and Urzì, 1999; Ortega-Calvo et al., 1995; Lamenti et al., 2000; Gorbushina et al., 2002; Hallmann et al., 2011a). In the case presented here, the surface of the marble sculpture was affected by biodeterioration in two different ways. Heavy blackish stains covered the entire surface and single mineral particles were detached from the surface and became attached to the lichen plectenchyme. Thus, one may expect that both effects may be temporarily stopped or reduced by surface cleaning and other treatments. However, by far most of previous studies just show colonization at one time point, and the gradual decrease of the cleaning effect by recolonization has been rarely addressed so far. One may speculate that after (re-) colonization has completed, the microbial communities are stable for several years (‘‘old grown’’ biofilms), representing a snapshot of a ‘‘climax’’ community. Our study documents the re-colonization 1 year after cleaning treatment in comparison to the ‘‘old grown’’ biofilm on the same marble sculpture.

The green algal sequences in the ‘‘old grown’’ biofilm (sample B) and on the cleaned surface (sample A) belong all to Trebouxiophyceae; members of this green algal class are mostly aerophytic or soil algae (Friedl and Rybalka, 2012). Adaptations against desiccation and UV irradiation like thick cell walls, mucilage sheaths and Mycosporine-like amino acids (MAAS) are common for aerophytic algae (Karsten et al., 2005a, 2005b). The occurrence of the trebouxiophycean genera *Apatococcus*, *Desmococcus*, *Stichococcus*, *Diplosphaera* and *Trebouxia* on stone substrates is well known (Hallmann et al., 2011a; Ragon et al., 2012).

On the 98 % similarity level two OTUs of *Apatococcus* were detected; the phylogenetic analysis confirmed two distinct groups (cf. Fig. 6). The cell package forming green algae *Apatococcus lobatus* is the most common aerophytic alga (Gärtner, 1994), abundant on natural stone substrata in the urban environment (Rindi, 2007). The resistance of *Apatococcus* against pollution, desiccation and high UV radiation is mainly due to thick cell walls and typical cytosolic ‘‘stress metabolites’’ like the polyols ribitol and erythritol (Barkman, 1969; Gustavs et al., 2011). *Apatococcus* was detected in both samples (old grown biofilm and cleaned area). Hitherto, lichen symbiosis for *Apatococcus lobatus* could not be definitively proven (Gärtner and Ingolić, 1989), but a close association with fungal hyphae was observed (Edlich, 1936). *Desmococcus*, another cell packages forming

trebouxiophyte, but phylogenetically distant from *Apatococcus* (Fig. 6), a very common aeroterrestrial green algae (Rindi, 2007) was also detected on the cleaned surface.

The dominance of different phylotypes of the lichen photosymbiont *Trebouxia* on both surfaces may be surprising since lichen thalli were present in the old grown biofilm, but not detected on the cleaned surface. However, *Trebouxia* has been frequently described in terrestrial habitats even when identifiable lichens were absent (Gärtner, 1994; Macedo et al., 2009). Furthermore, soredia (asexual reproductive structures) were observed by SEM on the surface of a lichen thallus (Fig. 2b) and single *Trebouxia* cells were closely associated with fungal hyphae (cf. Fig. 2c). This lichenization without an identifiable lichen thallus, in particular as asexual propagules or thallus fragments, is common for *Trebouxia* (Ahmadjian, 1988). Thus, various OTUs of *Trebouxia* could be detected possibly because of these dispersal structures of different lichens, colonizing tree bark and stone monuments nearby the cleaned sculpture, while obvious lichen thalli were absent on the cleaned sampling site A.

The green alga *Stichococcus* and *Diplosphaera*, both affiliated to Prasiolales-clade, were also discovered on both sampling areas. *Stichococcus* and *Diplosphaera* are known as lichen photobionts (Thüs et al., 2011). In addition, *Stichococcus* is an ubiquitous and rapid colonizer known from nitrogen rich sites (Ettl and Gärtner, 1995). Since the monument is situated under a tree, bird droppings (Hallmann et al., 2011a) may contribute to favorable conditions for *Stichococcus*. Generally, these photosynthetic microorganisms cause staining by photosynthetic pigments and may be involved in stone decay by secretion of organic acids and indirectly by supporting growth of other microorganisms like fungi and bacteria (Ariño et al., 1997; Polo et al., 2010).

Interestingly, fungal OTUs markedly differed in our studied samples. Eight of 12 fungal OTUs belong to the classes Dothideomycetes and Eurotiomycetes, which include numerous rock inhabiting fungi involved in stone biodeterioration (Gorbushina et al., 1993; Wollenzien et al., 1997; Ruibal et al., 2009).

The most abundant OTU 14 on the cleaned surface (sample A) is related to *Sarcinomyces* sp. isolate MA 4760, next related to *Sarcinomyces petricola* (Sert et al., 2007; Wollenzien et al., 1997), whereas the most abundant OTU 19 retrieved from the old grown biofilm (sample B) is related to *Rhinocladiella* sp. MA 4765 isolated from antique marble in Turkey (Sert et al., 2007). The black filamentous *Rhinocladiella* is free living, but also parasitic on lichens (Harutyunyan et al., 2008): *Sarcinomyces* is affiliated to meristematic black yeasts.

Whereas *Sarcinomyces* sp. was totally absent in the old grown biofilm, small number of *Rhinocladiella* sp. clones were also found on the cleaned surface. Thus one may speculate that *Sarcinomyces* appears to be a rapid colonizer, but was presumably outcompeted in our old grown biofilm by other fungi like *Rhinocladiella*. Just two OTUs in addition to *Rhinocladiella* sp. were shared by both surfaces, with just one clone in either sample A or sample B: *Glyphium elatum* (normally on wood, non-lichenized) and one OTU included in the family Teratosphaeriaceae (associated with Eucalyptus leaf diseases and stem cankers).

Only present in the old grown biofilm was *Bulgaria inquinans*, a saprobiontic fungus on tree bark, a species of the order Capnodiales, an epiphytic black fungus on plant leaves and often associated with the honeydew. In addition, exclusive in the old biofilm were *Caloplaca demissa*, a lichen fungus, with cyanobacteria instead of green algae in the thallus and a species of the family Davidiellaceae (with some cryptoendolithic representatives; Selbmann et al., 2005; Hallmann et al., 2011b).

Only presented on the cleaned surface were free living fungi like *Cladosporium*, well known from marble (Wollenzien et al., 1995), *Exophiala aquamarina* (a black yeast), an uncultured Banisveld eukaryotic clone (next related culture *Rhodotorula aurantiaca*, a yeast) and *Cryptococcus albidus* (cosmopolitan, e.g. on plants).

Most fungi were affiliated to ‘‘black fungi’’. In these groups the black pigment melanin is incorporated in vegetative hyphae, which provides protection against high UV irradiance (Bell and Wheeler, 1986). It is reasonable to assume that, besides algal pigments, melanin contributes to the blackish color of the old grown biofilm. Black fungi are frequently found in conjunction with honeydew coverings: Typically, honeydew is secreted by plant-sucking aphids and scale insects (e.g. Auclair, 1963). When the plant is massively infested by these insects, the resulting honeydew coverings of surfaces located underneath the plant are colonized by black fungi (Hallmann et al., 2011a). The investigated marble sculpture was situated under a tree, which causes a favorable microclimate (protection against high irradiation and evaporation; e.g. Weber et al., 2007) and provides nutrients as honeydew, dead plant material and bird droppings for microbial growth. A continuous input from the canopy is also documented by powdery mildew fruiting bodies (chasmothecia) scattered on the surface of the lichen thalli (Fig. 3e). Powdery mildew is a fungal disease affecting the tree leaves (Glawe, 2008 and references therein).

Little experience has been collected so far on recolonization of stone surfaces by microorganisms after cleaning. Though directly after cleaning with biocides, no remaining organisms could be detected (Polo et al., 2010), a long-term experiment documented re-colonization after cleaning treatment over 6 years (Lamenti et al., 2000). However, just enrichment culturing was used in this approach, which will greatly alter the microbial community before analysis. Twelve months after cleaning *Coccomyxa* sp. was observed as the dominant first colonizer. Six years after cleaning treatment, the community was again identical to an unrestored statue with green algae like *Coccomyxa*, *Apatococcus* and *Stichococcus*.

Miller et al. (2008) analyzed the biofilm development on a stone surface under laboratory conditions, using a molecular approach. One month after inoculation of the cleaned surface with an active growing microbial culture a green layer was observed; fungi were observed after 3 months. The detected eukaryotic phototrophic microorganisms belonged to the genera *Stichococcus*, *Trebouxia*, *Myrmecia* and *Chlorella*.

In our study, we could show that the cleaned re-colonized surface differs with respect to the fungal community, but not with respect to the green algal community, 1 year after re-colonization started. Up to now, this effect appears to be unavoidable, if not repeated cleaning and/or biocide treatment is applied. It must be kept in mind that early re-colonizers of a cleaned surface might be even more deleterious with respect to biogenic weathering than an old grown biofilm community (cf. Gaylarde et al., 2012).

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Chapter 3:
Microbial diversity on a marble monument:
a case study

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Abstract

In the presented case study, ascomycete fungi and green algae on a marble monument were identified by comparisons of the 18S rRNA gene sequences, which were obtained from DNA either from environmental samples or from enrichment cultures. The organisms were found to be responsible for either black or green surface coverings on different areas of the monument surface. Most fungi were related to plant-inhabiting genera, corresponding to a heavy soiling of the marble surface with honeydew. Whereas green algae of the genera *Stichococcus*, *Chloroidium* and *Apatococcus* were found to be dominant in all samples, isolates of two additional genera were recovered only from enrichment cultures. A reference strain of *Apatococcus lobatus* and an isolate of *Prasiolopsis* sp. were investigated with respect to putative surface adhesive structures of the cell envelope. The *Prasiolopsis* cell walls were covered with a thin adhesive exopolysaccharide layer involved in biofilm formation.

Keywords: marble monument, biofilm, ascomycete fungi, green algae, cell wall, exopolysaccharide.

Introduction

Biodeterioration of dimension stone primarily affects material surfaces. Some endolithic organisms actively penetrate the surface and are also found in layers up to several millimeters underneath the surface (e.g. Ascaso et al., 1998; Hoppert et al., 2004a). Though several important pro- and eukaryotic organisms have already been recognized as deteriorative agents, up to now little is known about the whole microbial community (see Gorbushina, 2007 and Macedo et al., 2009 for review). It must be expected that a microbial biofilm is composed of at least several dozens of species. These various organisms act in different ways on the material; a whole spectrum of deteriorative activities may be expected: from neutral (or even protective, e.g. Zuo et al., 2005) to deteriorative by penetration of the surface (Warscheid and Braams, 2000; Kemmling et al., 2004). It remains difficult to assign the deteriorative activity to a certain microbial species within a biofilm. Microbial activity also varies over seasons and is influenced by rainfall, surface cleaning or input of nitrogen, phosphate and other essential compounds.

The present case study aims at the identification of organisms by two complementary approaches: analyses of 18S rRNA gene sequences (either directly from environmental DNA or via enrichment culture) and isolation of strains. The former approach is inevitable for elucidation of microbial diversity, since most (pro- and eukaryotic) microorganisms are unculturable. The latter, ‘‘classical’’ approach underestimates the diversity as it recovers too few of the actually present species, but has two advantages. It enables studying the physiology of organisms in pure culture and allows recovering and identification of species that are present on the surface in such low numbers of individuals that they escape DNA extraction and sequencing. These species may actually not have an important role in the biofilm at the time of sampling, but may be present as resting stages and become more abundant when the environmental conditions (e.g. insolation, humidity) change.

For the present case study, a marble sculpture, which is part of the monument ‘‘Gegendenkmal’’ (Fig. 1a; Hamburger Dammtordamm, created by Alfred Hrdlicka 1983-1986), was selected. The object is covered with grayish and black crusts of hitherto unknown origin as well as greenish (obviously algal) biofilms. The aim of this case study was the application of both molecular and enrichment/isolation approaches to identify the organisms and to characterize cell wall features that are possibly relevant to biofilm formation. Here, we focus on green algal and fungal organisms. Members of these groups

dominate the biofilm, from the first microscopic inspection of the surface, with by far highest biomasses.

Materials and methods

Sampling

The sampling site was in Hamburg Dammtor (53°33′43″N, 9°59′27″E). Samples (approximately 100 µl dry volume) were collected in May 2008 from an SSW exposed part of the sculpture called ‘‘Hamburger Feuersturm’’. The samples were taken from two sites as depicted in Fig. 1b, c. Areas of 1 cm^2 from a green surface covering (Fig. 1b, sample A) and from a dark gray/black covering (Fig. 1c, sample B), respectively, were scraped off with a sterile scalpel. The samples were stored at ambient temperature in sterile 2 ml reaction tubes.

Cultivation and isolation

For the cultivation of microalgae, small amounts of the biofilm samples were either directly plated on agarized (1.5 %) culture medium MIEB12 (Schlösser, 1994) in Petri dishes, or inoculated in 10 ml volumes of liquid MIEB12 medium in culture tubes (enrichment culture). Cultures were kept under continuous illumination (25 µmol photons m^{-2} s^{-1}, white fluorescent tubes) and 18 °C for 4 weeks. From the liquid cultures, 100 µl aliquots were then plated on solid MIEB12 medium and incubated under the same conditions as for liquid media. Single colonies of different appearance were selected and transferred on fresh agar plates until unialgal cultures were obtained.

A reference strain, *Apatococcus lobatus* SAG 2037, was taken from the Culture Collection of Algae (SAG; Georg-August-Universität Göttingen, Germany) and cultured on agarized or liquid Bold’s Basal medium with vitamins (Schlösser, 1994).

Nucleic acid extraction, PCR, cloning and sequencing

DNA was extracted from the environmental samples as well as from the liquid cultures. All steps were performed with sterilized (autoclaved, nuclease-free) reagents. For DNA extraction, equivalents of 20-50 µl packed cell volume, either obtained from the original biofilm or from liquid cultures, were used. The biofilm samples or pelleted cells were resuspended in 100 µl lysis buffer (Invisorb Spin Plant Mini Kit, Invitek, Berlin, Germany).

Fig. 1. The monument and macroscopically visible surface stains. (a) The monument ‘‘Gegendenkmal’’, created by Alfred Hrdlicka 1983-1986, located in Hamburg-Altona. The marble sculpture at the left is nearly completely covered with a dark gray/black stain. (b), (c) Part of the sculpture ‘‘Hamburger Feuersturm’’ with a green (b) and dark-grayish (c) surface stain, as marked by arrows. (d), (e) Thin petrographic sections perpendicular to the surface from an area as depicted in (c). Small fragments, embedded in a dark matrix, adhere to the surface (arrows, d). Microfractures along grain boundaries, filled with a dark matrix (arrows).

After one volume of resuspended cells was mixed with an approximately equivalent volume of glass beads (425-600 µm diameter; acid-washed beads, Sigma-Aldrich, St. Louis, MO, USA) and vortexed briefly, the cells were mechanically disrupted by shaking in a Mini-BeadBeater (Biospec, Bartlesville, OK, USA) in several intervals of 30 s and one interval of 50 s at 5.000 rpm. DNA was then extracted with the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) in the extraction buffers, following the manufacturer's instructions. Results were checked on a 1 % (w/v) agarose gel. Isolated DNA was stored at -20 °C until further processing. The eukaryote-specific primer combinations NS1/18L and NS1/LR 1850 were used (Table 1) to amplify the 18S and the 18S with adjacent ITS1/5.8S/ITS2 regions of rRNA gene, respectively.

About 30 ng of the extracted DNA was used as template. The amplification reaction mixture (50 µl) contained each deoxynucleotide triphosphate at a concentration of 0.1 mM, 5 µl of tenfold concentrated reaction buffer, 2 mM $MgCl_2$, 0.2 µM primers, 2 U of Taq DNA polymerase (reagents and manufacturer's protocol: Bioline, Luckenwalde, Germany) and 4 % (v/v) dimethyl sulfoxide (DMSO)-solution. Polymerase chain reaction was carried out on a PTC 200 thermocycler (MJ Research, Waltham, MA, USA) using the following program for the primer pair NS1/LR1850: initial denaturation at 95 °C for 5 min, followed by 33 cycles of denaturation at 94 °C for 40 s, annealing at 52 °C for 90 s, extension at 72 °C for 90 s, followed by 6 cycles of denaturation at 94 °C and final extension at 72 °C for 2 min. For the primer pair NS1/18L, denaturation was for 1 min, annealing for 45 s, extension for 3 min, the additional 6 cycles were omitted and final extension was for 7 min. The PCR products were purified using the Invisorb Spin PCRapid Kit (Invitek, Berlin, Germany). Aliquots (2 µl) of purified amplicons were analyzed by electrophoresis on a 1 % (w/v) agarose gel. Cloning of the PCR product was performed with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and the pCR2.1-TOPO vector.

Table 1. Primers used in this study**.**

Primer	Sequence (5'-3')	References
NS1 (forward)	GTAGTCATATGCTTGTCT	White et al. (1990)
18L (reverse)	CACCTACGGAAACCTTGTTACGACTT	Hamby et al. (1988)
LR1850 (reverse)	CCTCACGGTACTTGTTC	Friedl (1996)
M13F (forward)	TGTAAAACGACGGCCAGT	Invitrogen
M13R (reverse)	GGAAACAGCTATGACCATG	Invitrogen
895R (sequencing primer)	AAATCCAAGAATTTCACCTC	SAG

Ligations were transformed into competent cells of *Escherichia coli* TOP 10, as supplied by the manufacturer. In the plasmid screening, white *E. coli* colonies containing correct DNA insertions were identified by direct amplification of the inserted DNA fragment with a vector-specific primer set M13F/M13R (Table 1).

Clones were cultivated overnight in LidBac reaction tubes (Qiagen, Hilden, Germany) with 1 ml LB medium containing 100 µg ampicillin. Plasmid DNA was prepared from the clones with a NucleoSpin-Plasmid kit (Macherey and Nagel, Düren, Germany) following the manufacturer's instructions.

Sequencing reactions were performed with a Dye Terminator Cycle Sequencing v3.1 kit (Applied Biosystems, Darmstadt, Germany) and an ABI Prism 3100 (Applied Biosystems) automated sequencer. All clones were sequenced with the 18S sequencing primer 895R (Table 1) resulting in about 600 bp sequences. Sequences were processed using the sequence analysis program SeqAssem (Sequentix, Klein Raden, Germany) and manually aligned using the program BioEdit v7.0.5.3 (Meusnier et al., 2008). The final sequences were compared by BLASTn analysis [National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov].

Light and electron microscopy

For fluorescence light microscopy, cells taken from unialgal or pure cultures were marked with concanavalin A, coupled to fluorescein isothiocyanate (Con A-FITC; Sigma-Aldrich). The dye was applied in 50 mM potassium phosphate buffer, supplemented with 10 µM magnesium chloride and 10 µM calcium chloride at a dilution of 1/1,000 of the original stock solution. The sample was inspected under a fluorescence light microscope (Axioscope, Zeiss, Göttingen, Germany; excitation wavelength 495 nm, emission wavelength 517 nm, Zeiss filter set 09).

Petrographic thin sections of approximately 30 µm in thickness were performed according to established procedures (Adams et al., 1984) and visualized by standard bright field microscopy.

For electron microscopy, cells from unialgal or pure cultures were harvested by centrifugation at 10.000 x g, resuspended in 50 mM potassium phosphate buffer, chemically fixed in 0.5 % (w/v) formaldehyde and 0.3 % (w/v) glutaraldehyde solution for 90 min at 0 °C, dehydrated in a graded methanol series and embedded in Lowicryl K4M resin (Roth et al., 1981; Hoppert and Holzenburg, 1998). Resin sections of 80-100 nm thickness were cut with glass knives. Localization studies were performed with the lectin

concanavalin A (Sigma-Aldrich), coupled to colloidal gold (Con A-Gold), as already described (Kämper et al., 2004). In brief, sections were incubated on drops of dilutions (1/10, 1/100, 1/1,000) of the Con A-gold marker for 90 min, then washed for 5 min per step for three times on drops of PBS containing 0.01 % (v/v) Tween 20. Finally, the sections were stained with 0.5 % (w/v) phosphotungstic acid, pH 7.0, for 3 min. Electron microscopy was performed in a Zeiss EM 902 transmission electron microscope (Zeiss SMT, Oberkochen, Germany), equipped with a 1 K digital camera, at 80 kV acceleration voltage and at calibrated magnifications.

Results

Diversity of fungal and algal organisms

The monument ‘‘Gegendenkmal’’ consists of several bronze and marble sculptures. In this study, the sculpture ‘‘Hamburger Feuersturm’’, made of Carrara marble, was under investigation. The sculpture showed a mixture of greenish and grayish/black stains (Fig. 1), as well as green layers on the fracture surfaces of chips and scales (cf. Warscheid and Braams, 2000). The sculpture was manufactured from Bianco Carrara C, a pure calcite marble, as determined by X-ray diffractometry (data not shown).

Thin petrographic sections, perpendicular to the surface, show open grain boundaries between the calcite crystals in the marble microstructure (Fig. 1d, e), which illustrates the increased porosity and a slight sugar-like crumbling of the marble surface. The grayish stain, mainly on the top parts of the marble sculptures, was the most obvious feature (Fig. 1a, c). In petrographic sections, the stain appears to be homogeneous, slightly red-brown and translucent. It encloses crystals on the marble surface (Fig. 1d) and infiltrates the surface along open grain boundaries (Fig. 1e). Though the cause of this heavy soiling could not be identified, it was observed that honeydew covered the sculptures in the affected areas. The honeydew was dripping off a plane tree canopy (*Platanus* x *hispanica*), placed directly above the monument, as well as a lime tree canopy (*Tilia platyphyllos*) nearby. Another obvious feature was represented by green stains of algal biofilms, primarily growing in cavities, where water cannot drain off fast.

To investigate the participation of microorganisms in these surface coverings, material from approximately 1 cm^2 surface was used for further processing. Green layers under scales were not enclosed in this study, because they represented just a small fraction of the (putatively) biogenic stains. Sample A was taken from a green algal biofilm (Fig. 1b) and

sample B from the black covering as depicted in Fig. 1c. The samples were first inspected by light microscopy to verify the presence of dominant species. Actually, green algal (sample A) and fungal (sample B) morphotypes dominated the microbial biomass of the biofilm (Fig. 2a, b). Thus, our further studies aimed at the identification of the species from both these groups. Most of the algal morphotypes correspond to *Apatococcus* and *Chloroidium ellipsoideum* (Darienko et al., 2010). No stratification of the biofilms could be observed: filamentous fungi (if present) and green algae were interwoven in a homogeneous surface covering. In enrichment cultures, inoculated with samples from the original biofilm, besides *Chloroidium*-morphotypes, rod-shaped *Stichococcus*-like cells dominated (Fig. 2c). Unialgal cultures could be obtained for some of the dominant *Stichococcus* and *Apatococcus* like morphotypes (cf. Fig. 3a, b). In addition, a xanthophyte alga (Fig. 3c) and a *Prasiolopsis* (*Pseudopleurococcus*) -like morphotype (Fig. 3d; see below) could be isolated.

Sequences obtained from clone libraries of environmental DNA samples also recovered *Stichococcus* (*S. mirabilis* related) and *Apatococcus*, however, failed to detect *Prasiolopsis* and xanthophytes (Table 2). Instead, *Trebouxia* (a frequent lichen alga) was found. Besides the dominating *Stichococcus* spp. algae, a variety of ascomycete fungi was found exclusively in sample B. Among the fungi, members of the ascomycete genera, *Batcheloromyces*, *Teratosphaeria*, *Thelocarpon* and *Sarcinomyces*, were recovered, each with more than one clone. *Batcheloromyces*, *Teratosphaeria* and *Guignardia* are known as plant-inhabiting ascomycetes (Crous et al., 2004). *Thelocarpon* represents a lichenicolous ascomycete genus. The genus *Sarcinomyces* describes black yeasts from various habitats (Cooke, 1961).

The clone libraries from the enrichment cultures showed lower diversities. *Stichoccoccus bacillaris*-like algae were dominant (Table 3) and no fungal organisms could be detected. It has to be stated, nevertheless, that not all organisms could be assessed by the clone library analysis. The observed xanthophycean algae as well as *Prasiolopsis*, which were observed in the cultures, were not recovered by clone libraries from environmental samples. The identity of the *Prasiolopsis* genus was confirmed by 18S rRNA gene sequencing of preparations obtained from unialgal cultures.

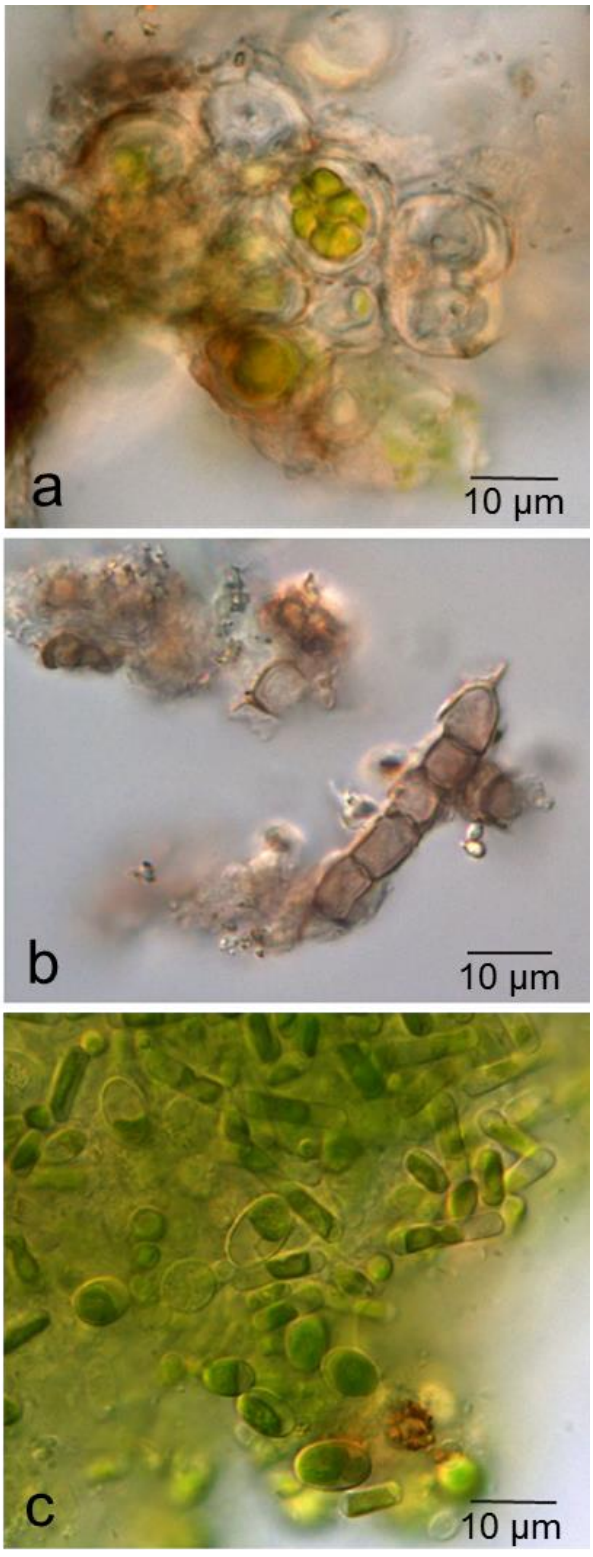

Fig. 2. Microscopic analysis of environmental samples and enrichment cultures. (a) Agglomeration of coccoid algae taken from the marble surface. (b) Ascomycete fungal morphotype (putative conidial fragment) taken from the surface. (c) Unicellular green algal morphotypes in an enrichment culture.

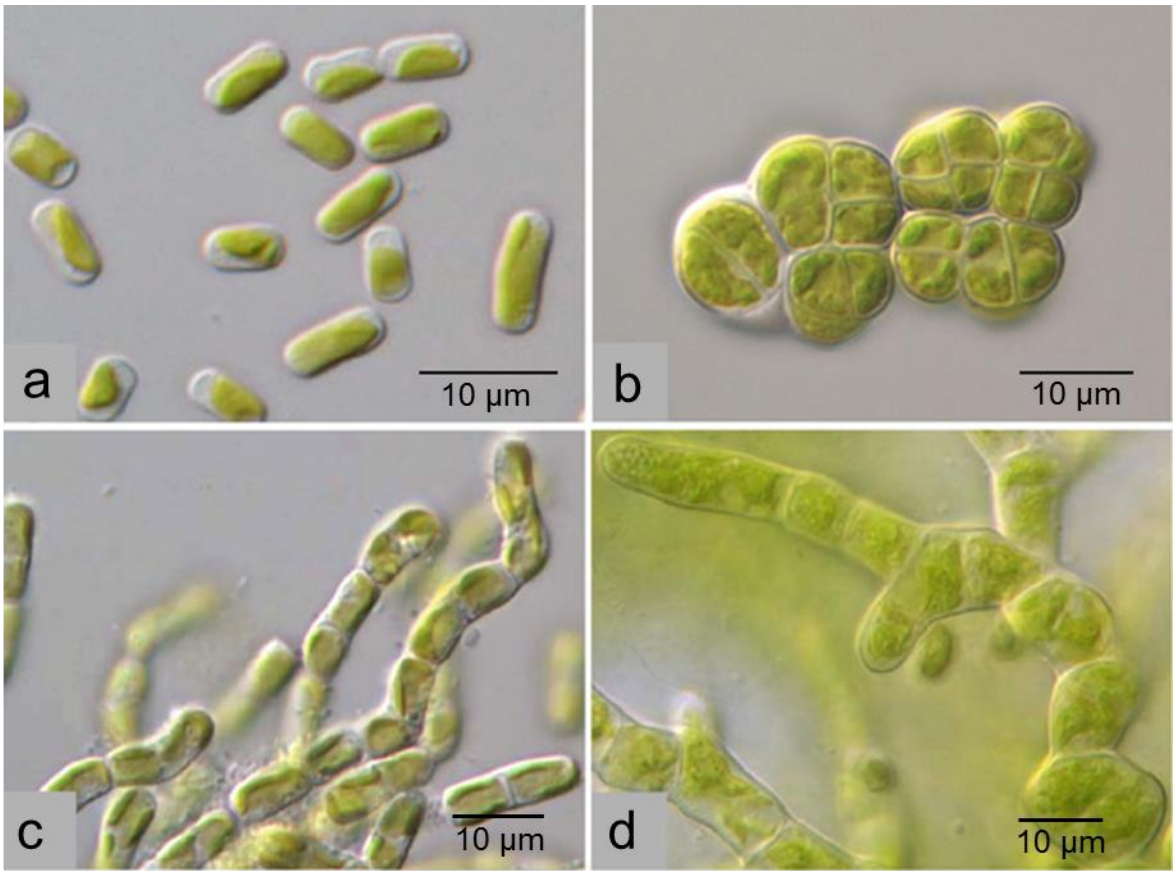

Fig. 3. Morphotypes of the investigated algal strains. (a) *Stichococcus* sp., (b) *Apatococcus lobatus* SAG 2037, (c) a Xanthophycean algae, (d) *Prasiolopsis* sp.

Cell surface features of selected species

The branched filamentous *Prasiolopsis* was selected for further morphological studies. The organism was compared with a contrasting morphotype, represented by *Apatococcus lobatus* SAG 2037, which forms irregular packages consisting of several dozens of cells (Figs. 3b, 4a). These algae represent the most different biofilm morphotypes in this study, i.e., unicellular coccoid and branched filamentous (Figs. 3b, d, 4).

With respect to biofilm formation, we were particularly interested in cell surface features that are involved in adhesion, such as secreted polysaccharides (e.g. Tsuneda et al., 2003). In search of such features, polysaccharide-specific stains were applied (Figs. 4, 5). In ultrathin sections of the algal cell walls, a thin irregular layer on the surface of the *Prasiolopsis* cell walls was observed (Fig. 4e, arrows), whereas the cell walls of *Apatococcus* did not show any additional layers (Fig. 4b). To detect possible heteropolysaccharides of the cell envelope, staining with labeled concanavalin A (Con A) was applied. In both algae, the cell periphery showed certain fluorescence signals

(Fig. 4a, d). Localization with Con A-gold revealed, at electron microscopic resolution, a scattered distribution of gold markers over the whole cell wall section of *Apatococcus* (Fig. 4c), and a very distinct marked outer layer in *Prasiolopsis* (Fig. 4f). This accounts for a thin layer of exopolysaccharides (EP) in *Prasiolopsis*. This layer appears to be involved in adhesion of separate *Prasiolopsis* filaments as depicted in Fig. 5. Here, the cells adhere to each other by the thin EP layer (Fig. 5b).

Table 2. 18S rRNA gene analysis of clones obtained from environmental samples.

Closest relative species	Number of clones (sample A)	Number of clones (sample B)	% sequence similarity to closest relative species
Algae			
Stichococcus mirabilis	10	24	98-99
Uncultured *Trebouxia* sp.	1	1	97-98
Apatococcus sp.	2	–	98-99
Stichococcus sp.	1	1	99
Stichococcus jenerensis	1	–	98
Chloroidium mirabilis	1	–	98
Stichococcus bacillaris	1	–	96
Acomycete fungi			
Batcheloromyces proteae	–	5	98-99
Teratosphaeria microspora	–	3	96
Thelocarpon laureri	–	3	95-97
Sarcinomyces sp.	–	2	93-97
Guignardia mangiferae	–	1	100
Phaeoramularia hachijoensis	–	1	98
Phialophora sp.	–	1	98
Conisporium perforans	–	1	97
Spencermartinsia sp.	–	1	97
Xenomeris raetica	–	1	97
Symbiotaphrina kochii	–	1	97
Mycocalicium polyporaeum	–	1	96
Aureobasidium pullulans	–	1	95
Harpidium rutilans	–	1	95
Pseudofusicoccum stromaticum	–	1	93

Table 3. 18S rRNA gene analysis of clones obtained from enrichment cultures.

Closest relative species	Number of clones (sample A)	Number of clones (sample B)	% sequence similarity to closest relative species
Algae			
Stichococcus bacillaris	21	12	98–100
Chloroidium ellipsoideum	4	2	98–100
Chloroidium angustoellipsoideum	3	1	99
Stichococcus mirabilis	–	3	98
Stichococcus deasonii	2	–	100

Discussion

Our case study aims at the elucidation of surface stains caused by microbial impact. Besides general climatic features (such as precipitation, insolation or temperature), especially local factors (the effect of honeydew) determine the biofilm formation and its taxonomic composition.

The sampling site in Hamburg experiences an oceanic climate, with average annual precipitation of 774 mm and an average annual temperature of 9 °C. The average temperature in May 2008, however, was approximately 15 °C (3 °C higher than the long-time average for May). Precipitation was just about 25 % of the long-time average for May (54 mm) (data were taken from Deutscher Wetterdienst, Offenbach). Thus, the biofilms developed under relatively warm and dry conditions. It is, however, still difficult to draw a relation between climate and the detected organisms: most of the detected species are distributed worldwide, with seemingly broad ecological amplitude, a finding typical for most microorganisms known so far (c.f. Finlay and Esteban, 2004). Thus, a ‘‘biogeography’’ of microbial species is yet to be revealed (Hedlund and Staley, 2004; Martiny et al., 2006). From the data presented here, however, one feature is obvious. Molecular analysis of 18S rRNA gene clone libraries from sample B revealed a high diversity of ascomycete fungi, as compared with sample A, dominated by algae (Table 2). Sample B was taken from an area intensively soiled by honeydew. Sample A was taken from a cavity (Fig. 1b), where, due to the complex geometry of the sculpture, immediate soiling with honeydew can be excluded. Here, the photoautotrophic algae are dominant. These cavities will retain rainwater for longer time than the exposed surfaces. Rainwater may also transport some dissolved carbohydrates (see below) from the honeydew layer to

the cavity but, in toto, the conditions are obviously more favorable for algae than for ascomycetes.

Intriguingly, among the most frequently found clones from sample B, the closest related species are known as plant associated or plant pathogenic. *Batcheloromyces proteae* and *Teratospora* (*Mycosphaerella*) microspora are (opportunistic) pathogens, which are causative agents of black leaf spot disease (Crous et al., 2004). Also *Spencermartinsia* sp. is a plant-inhabiting fungus and an opportunistic phytopathogen (Phillips et al., 2008), as well as *Xenomeris raetica*. Members of the genus *Xenomeris* are possibly infectious agents of tree canker (Jasalavich et al., 2000).

Phialophora comprises plant- and human-pathogenic species, as well as saprophytic, wood-decaying non-pathogens (Abliz et al., 2004). *Guignardia magniferae* is an endophytic, but not necessarily a plant-pathogenic, fungus (Suryanarayanan et al., 2004). Undoubtedly, the plant- associated fungi were transferred from the tree canopies to the marble surface, by honeydew droplets. Honeydew is a carbohydrate-rich secretion mainly consisting of monosaccharides, as well as the trisaccharide melezitose (*O*-*α*-*D*-glucopyranosyl-(→3)-*O*-*β*-*D*-fructofuranosyl-(2→1)-*α*-*D*-glucopyranoside), which can be easily used by fungi and other microorganisms as growth substrates (Fischer et al., 2002). The concentration of amino acids in honeydew is relatively low (in the range of 3-20 nmol/µl), which is a growth-limiting factor. However, the overall nitrogen input (also from atmospheric sources) is sufficient for the development of plant-associated fungi: such as other oligotrophic fungi, they are well adapted to the plant biomass with wide C/N ratios (Wainright et al., 1993 and references therein).

Massive colonization of black-pigmented ascomycete fungi (‘‘black fungi’’, Dematiaceae) has been frequently observed in conjunction with honeydew coverings of surfaces (e.g. Crozier, 1981; Gerson, 1975). On leaves of affected plants, colonization is referred to as sooty mold Sooty mold is also a common disease on plane and linden trees (Hughes, 1976). Among other genera, also *Aureobasidium* species have been described for the normal phyllosphere as well as for sooty mold. *Aureobasidium* is not just a plant-associated ascomycete. Members of this genus, as well as the detected *Sarcinomyces*, frequently occur on rock and dimension stone surfaces (Simonovicova et al., 2004; Wollenzien et al., 1997). Organisms of this group have been identified as deteriorative rock-dwelling agents on natural and dimension stone (Gorbushina et al., 1993; Gorbushina and Krumbein, 2000). The black pigmentation is attributed to the ultraviolet-protective melanin (Bell and Wheeler, 1986).

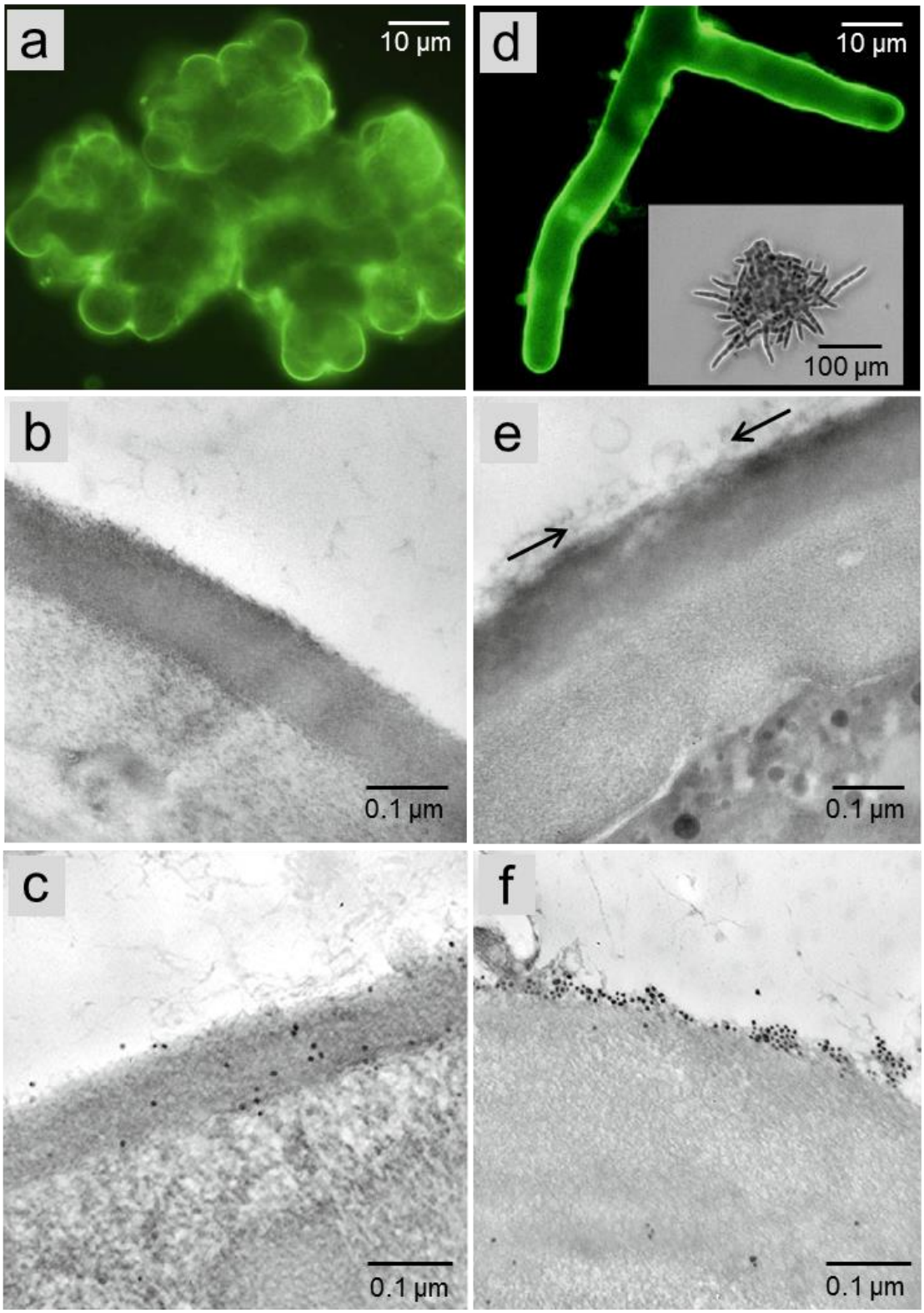

Fig. 4. Light and electron microscopy of lectin-labeled cells. (a) Fluorescence light microscopy of a Con A-FITC- labeled *Apatococcus* aggregate. (b) Ultrathin section of an *Apatococcus* cell wall. (c) Con A- gold labeled ultrathin section of an *Apatococcus* cell wall. Dark dots represent the colloidal gold marker. (d) Fluorescence light microscopy of Con A-FITC- labeled *Prasiolopsis* filaments. The inset shows a typical aggregate (phase contrast image). (e) Ultrathin section of a *Prasiolopsis* cell wall. The thin exopolysaccharide layer is marked by arrows. (f) Con A gold-labeled ultrathin section of a *Prasiolopsis* cell wall.

In fungi, melanin is synthesized via different pathways, frequently from acetate via 1,8-dihy-droxynaphthalene as intermediate. The resulting phenolic polymer is deposited in- or outside the fungal cell wall (Bartnicki-Garcia, 1968). Hence, the dark stain is difficult to handle. Though superficial soiling, even black gypsum crusts on top of a surface are removable with mild chemical or biological agents (e.g. Polo et al., 2010), the organisms and stains may also penetrate the genuine marble surface (cf. Fig. 1e). When these cell wall remnants cannot be removed mechanically, or by application of mild detergents, rather harsh chemical treatment, e.g. with hydrogen peroxide as strong oxidant, is necessary to destroy the melanin (Korytowski and Sarna, 1990).

According to the clone library data, the group of plant-associated ascomycetes dominates over the rock-inhabiting genera. Though even sequencing clone libraries to saturation (which was not done here) does not reflect the real species diversity exactly (Jeon et al., 2008), we find in our case study a clear tendency for the presence of ‘‘allochthonous’’, not genuinely rock-inhabiting fungi. The organisms grow in the honeydew cover on the marble sculpture, whereas the ‘‘autochthonous’’ organisms do not benefit from the honeydew in the same way. In contrast, the algal species of the genus *Stichococcus* are typical colonizers of stone surfaces (cf. e.g. Michailyuk, 2008). It is noticeable that, though the algae are photoautotrophic, they may also benefit from external organic nutrients, especially from sucrose (e.g. Samejima and Myers, 1958).

Growth enhancement by carbohydrates has also been shown for *Stichococcus mirabilis* (Mattox and Bold, 1962). This may be one reason for the abundance of this algal species in a biofilm dominated by diverse fungi. Generally, the most abundant algal species were small unicellular morphotypes, known as pioneer organisms (e.g. Garty, 1992; Bellinzoni et al., 2003). Besides these organisms, others must be present in very low numbers of individuals, as a ‘‘seed bank’’ inside the biofilm (Table 3). Hence, their sequences were not present in the clone libraries, derived from environmental DNA. They will become important when environmental conditions change (which is, in fact, the case during enrichment of organisms from biofilm samples in a liquid culture). Some species may even not be detected in clone libraries of enrichment cultures, such as, in our example, *Prasiolopsis* sp., a green alga that could be obtained in unialgal culture. *Prasiolopsis* is a peculiar subaerial alga living on rock or tree bark with multiseriate filamentous or pseudo-parenchymatous thalli (Karsten et al., 2005). Branched filaments are well adapted to those environments where tiny paths and small cavities, as well as impassable (crystalline) particles form a more or less solid substratum (e.g. Ritz and Young, 2004).

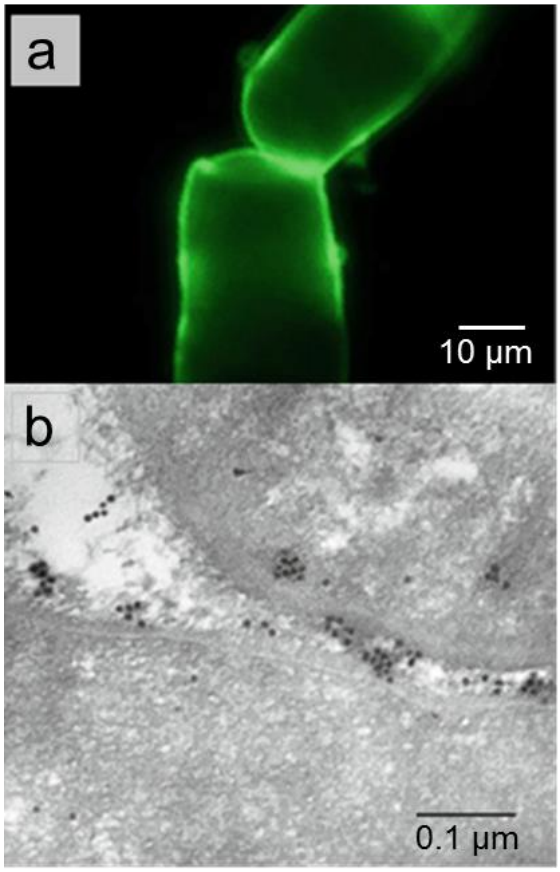

Fig. 5. Agglutination of *Prasiolopsis* filaments. (a) Fluorescence light microscopy of two attached filaments. (b) The same situation as depicted in a visualized by electron microscopy. Two attached filaments agglutinate via a thin exopolysaccharide layer, labeled with the Con A-gold maker.

Moreover, the algal exopolysaccharides (EP) facilitate the attachment to a surface as well as agglutination of organisms to each other. Up to now, mainly thick algal EP layers in aquatic biofilms have been described (see Sutherland, 2001 for review), where green algae and cyanobacteria are covered with a thick polysaccharide layer. It is also known that EP stabilizes terrestrial (soil) biofilms such as microbiotic soil crusts (Hoppert et al., 2004b; Bowker et al., 2008). The role of EP on dry, solid surfaces is much less known, though it is obvious that the organisms also produce these extracellular polymers. For detection of specific oligosaccharides in EP, marker techniques based on the binding of lectins to certain oligosaccharides stretches are used. The lectin concanavalin A binds to oligomannose-type N-glycans. These motifs are common in cell wall polysaccharides and especially in EP of a variety of organisms, including cyanobacteria and eukaryotic algae (Mehta and Vaidya, 1978; Tien et al., 2005).

The *Apatococcus* strain does not show a layer adjacent to the cell wall, though the rigid wall itself exhibits a dispersed labeling (Fig. 4c). Heteropolysaccharides of different proportions are common in algal cell walls (Takeda and Hirokawa, 1984; Okuda, 2002) and can also be expected for *Apatococcus*. *Prasiolopsis*, in contrast, forms a thin layer of

just several tens of nm in thickness, distinctively labeled with the concanavalin A-lectin (Fig. 4e, f). Compared with other EP layers from eukaryotic algae (e.g. Leppard, 1995), *Prasiolopsis* exhibits even in the hydrated state only a thin EPS coating. This layer may not have more than a gluing function. Up to now, there are no data concerning the interaction between the filaments and a material surface, but it is obvious that single filaments agglutinate via the thin EP layer (Fig. 5). This may be an important feature for the formation of an interwoven meshwork of branched filaments.

Conclusion

In our case study, we examined the microbial diversity on a monument contaminated by honeydew. Remarkably, typical rock-inhabiting fungi appear to be less relevant than plant-inhabiting fungal microorganisms. Less contaminated areas of the sculpture exhibited typical green algal biofilms. Among the algae, unicellular *Stichococcus*-, *Chloroidium*- and *Apatococcus*-related strains were dominant in environmental samples and in enrichment cultures. Other algae, like *Prasiolopsis*, could also be isolated.

These results show the high relevance of an external carbon source (honeydew) for surface colonization by ascomycete fungi and, hence, formation of a dark-stained biofilm layer. The dark surface stain can only be avoided by removal of the carbon source, e.g. the honeydew, which may be achieved by suitable pest control. Also, an appropriate shaping of the canopy may lead, in this case, to an effect: parts of the sculptures are presently positioned in a way that they are placed rather under the periphery of the tree canopies. Thinning of the crown may not completely prevent, but reduce, the impact of down-dripping honeydew.

Acknowledgments

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Chapter 4:
Biodiversity of green phototrophic biofilms on artificial hard surfaces: a case study of aero-terrestrial algae in an urban environment using molecular approaches

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Abstract

Air-exposed green algae dominated biofilms on artificial hard substrates of urban environments represent an extreme habitat with respect to ever diminishing moisture coupled with strong fluctuations in temperature and PAR/UV irradiation. In Middle Europe they are common leading to undesired optical effects and accelerated biocorrosion of house facades, concrete walls and outdoor plastic objects. In a case study 18S rRNA gene cloning/sequencing directly from the biofilms of eleven urban sites with various substrates revealed the prevalence of at least two distinct Operational Taxonomic Units (OTUs) or species of *Apatococcus,* three OTUs of *Trebouxia* and several microfungi, but no sequence counterparts for the additional morphotypes visible by microscopy of the biofilms were recovered. Also the phylogenetically distinct *Desmococcus* was not detected. For one study site, the polyethylene surface of a compost waste container, six more OTUs from five genera of Trebouxiophyceae as well as *Klebsormidium* were recovered when crude cultures were established to suppress growth of *Apatococcus* in favor of other species. Species so far reported only from Antarctica, *Coenochloris signiensis* and *Pabia signiensis*, or the tropics, *Heveochlorella roystonensis*, were detected in agreement with a world-wide distribution of the areo-terrestrial green algae as well as representatives of a new genus within the *Watanabea*-clade of Trebouxiophyceae. In addition, isolates established from the exemplar site exhibited structures probably facilitating adhesion and/or serving to protect the cells from drought. The genetic diversity of the biofilms contrasted their morphological uniformity; both may be the results of constraints effective in these extreme habitats.

Keywords: biofilm, extreme habitat, aero-terrestrial microalgae, *Apatococcus, Desmococcus,* Trebouxiophyceae.

Introduction

In many urban areas in Middle Europe green phototrophic biofilms, dominated by green algae, colonize various types of substrates such as artificial building material (e.g. roof tiles, house facades, concrete wall, natural building stone), glass, metal, or plastic surfaces (e.g. glass roofs, street signs, waste containers) (Rindi, 2007; Karsten et al., 2007) which may also comprise a variety of mosses, fungi, lichens and bacteria (Gorbushina, 2007; Hallmann et al., 2015). Several hazards may be caused by the green algal biofilms on surfaces and, therefore, to study their biodiversity may be a first essential prerequisite to avoid the appearance of these biofilms. Biofilms lead to biocorrosion of material which is mainly driven by the excretion of organic acids and, hence, dissolution and complexation of calcium and magnesium ions (Welton et al., 2003; Karsten et al., 2005a). Also the biodeterioration due to biofilms of synthetic polymers, like epoxy resin applied as material for statues or for restoration applications, has recently been described (Cappitelli and Sorlini, 2008; Pangallo et al., 2014). The extent of the biocorrosion may depend on the algal life style, e.g. endolithic or epilithic growth (Hoppert et al., 2005; Horath and Bachofen, 2009), and, thus the composition of the algal community on a surface. Algal biofilms contribute to staining and soiling of the surface due to the production of red (caused by carotenoids) and green (chlorophylls) pigments and, thus, may led to undesired staining of air-exposed surfaces in an urban environment, e.g. house facades. The moisture regimen of facades strongly influences the establishment and growth of algal biofilms. In the past few years prolonged frost-free periods in winter promoted growth of aerophytic microalgae on artificial hard substrate surfaces in the Northern hemisphere. Frequently, also heat insulation of facades may cause a moisture regime which in addition promotes the establishment of the green algal biofilms (Karsten et al., 2005a).

In terrestrial habitats, green algae are among the most widespread eukaryotes (Darienko and Hoffmann, 2003; Rindi, 2011) and have already been recognized since the beginning of systematic research on algae (e.g. Agardh, 1824; for further references see Gärtner, 1994; Rindi, 2007). Aerophytic algae, i.e., algae which are dispersed by airflows, exhibit a variety of adaptations to their often extreme habitats. Dry periods, with temperatures on air-exposed surfaces in summer reaching up to 50 °C and higher and in winter below 0 °C, require an adaptation of the algae mainly against desiccation. These adaptations may be manifested in highly thickened cell walls (e.g. the *Apatococcus/Desmococcus* morphotype and *Klebsormidium* spp.) or gelatinous sheaths

formed by extracellular polymeric substances (EPS), e.g. in *Coccomyxa* (Karsten et al., 2005a; Holzinger and Karsten, 2013; Karsten et al., 2014). These structures may enable the green algal cells to store water for a certain time period and help to withstand high osmotic stress during desiccation. The accumulation of EPS as a protection against desiccation has already been reported (Shepherd and Beilby, 1999; Hoppert, 2003). Equally important are mechanisms protecting against high radiation, such as the accumulation of carotenoids and the formation of MAAs (mycosporine-like amino acids; Karsten et al., 2005b). Moreover, resting stages such as spores persist for years (Karsten et al., 2005a). In addition, also the chemical properties of the substratum substantially influences the settlement of specific algae (Darienko and Hoffmann, 2003; Rindi, 2007).

Members of various green alga genera, e.g. *Apatococcus*, *Chlorella*, *Desmococcus*, *Klebsormidium*, *Stichococcus* and *Trebouxia* are known as the most common representatives of aerophytic microalgae (Gärtner, 1994; Karsten et al., 2005a; Rindi et al., 2010). In spite of these general findings, knowledge on the algal diversity of the green algal biofilms is still in its infancy (Karsten et al., 2005a; Rindi, 2007). Up to now, the green biofilms have mostly been studied by microscopy, i.e. recognition of certain morphotypes. Only a few have been isolated and cultured (Darienko and Hoffmann, 2003; Uher, 2008; Khaybullina et al., 2010; Neustupa and Štifterová, 2013). However, most green algal species cannot be correctly identified by microscopy of the biofilm because their diagnostic features may appear only in culture and are hard to recognize for the non-specialist. In addition, some biofilm algae may be difficult to isolate into culture. Therefore, the study of molecular characters is required, i.e. to identify the biofilm organisms using DNA sequence comparisons. The taxonomic composition of other terrestrial microalgal communities, e.g. Biological Soil Crusts, rock and building stone surfaces have been investigated using molecular phylogenetic markers (Lewis and Lewis, 2005; Horath and Bachofen, 2009; Büdel et al., 2009; Cuzman et al., 2010; Hallmann et al., 2013a). Though only a fraction of the algal diversity may be detected by culturing of samples, algal taxa may be enriched that are represented by only few individuals in the original samples - which may otherwise remain undetectable even with molecular techniques.

In an attempt to study the algal diversity of green phototrophic biofilms using 18S rRNA gene sequence comparisons we took biofilm samples from eleven localities representing various substrates within the urban environment of the city of Göttingen, Germany (Table 1). Due to relatively high moisture with frequent rainfalls and cool

average air temperatures over the whole year green algal dominated biofilms are well developed on various air-exposed artificial substrates at different heights within the city of Göttingen. Microscopy of the biofilm samples revealed the dominance of the common *Apatococcus/Desmococccus* morphotype together with *Trebouxia* spp. over much less abundant smaller round or ovoid cells with a *Chlorella*- or *Coccomyxa*-like appearance and sometimes also round green algal cells covered by mucilage. Because the culture-independent approach of PCR amplification, cloning and sequencing directly from DNA extracts of the biofilms revealed a just very poor diversity of the biofilms, i.e. representatives of *Apatococcus* and *Trebouxia*, we selected one locality as an example to study the biofilm algal community in more detail, i.e. also using crude (enrichment) cultures and green algal isolates. The latter efforts revealed a diversity of eleven molecular Operational Taxonomic Units (OTUs) or species from at least six genera of Trebouxiophyceae (Chlorophyta) apart from *Klebsormidium*.

Materials and methods

Green phototrophic biofilms from eleven sites with air-exposed surfaces of various artificial substrates were collected within the city limits of Göttingen, Germany, during the year 2006 (Table 1). The biofilms were openly exposed to the environment and situated at least at height of approximately 1.5 m above ground in a horizontal position. Only site MA was covered by vegetation at about 20 cm above ground and, therefore, shaded and with more moisture than the other sites. The biofilm samples were scraped off with a sterile scalpel and stored in 2 ml reaction tubes. One site, a polyethylene surface of a compost waste container (GOG, Fig. 1a, b), was selected as an exemplar site to study the algal composition of the biofilm also by crude cultures. For them small portions of the biofilm samples were suspended into a glass tube with 10 ml liquid $MIEB_{12}$ medium (http://www.uni-goettingen.de/de/184982.html). For isolation of microalgae a small amount of the biofilm sample was spread onto $MIEB_{12}$ agar plates. The crude cultures and agar plates were incubated at 18 °C on a 14:10 hour light:dark cycle at 25 µmol photons m^{-2} s^{-1} of white fluorescent light for 4 weeks. From agar plates colonies were selected and purified by repeated transferring on fresh agar media until unialgal cultures were obtained. The isolates were then maintained on agar slants. Representative isolates were accessioned by the *Sammlung von Algenkulturen der Universität Göttingen* (SAG) under the strain numbers SAG 2359, SAG 2373, SAG 2374 and SAG 2375 (Table 2).

Table 1. The locations and substrates used for the study of biofilm composition using 18S rRNA gene clone libraries directly from the biofilm samples, number of retrieved clones and their distribution on the recovered green algal genera.

Biofilm location and clone library code	Substrate	Location Coordinates	Total no. of clones per library	*Apatococcus*	*Trebouxia*	*Chloroidium*	Trebouxiophyceae	*Pabia*	*Coccomyxa*	*Stichococcus*	Others
GOG	polyethylene surface (waste container)	51° 32.089'N, 09° 56.393'E	122	94	8						fungi (20)
SK	plastic surface (eletricity connection box)	51° 32.309'N, 09° 56.222'E	27	3	16						fungi (8)
SKL	plastic surface (eletricity connection box)	51° 32.435'N, 09° 54.029'E	31	11	3			1			fungi (16)
EGU	wooden fence railing	51° 32.665'N, 09° 56.903'E	45	45							
GSW	metal road sign	51° 32.125'N, 09° 56.403'E	21	10	11						
GLK	metal lamppost	51° 32.449'N, 09° 54.031'E	39	39							
GLG	metal lamppost	51° 32.515'N, 09° 55.162'E	50	50							
GLB	metal lamppost	51° 32.200'N, 09° 56.165'E	50	48							fungi (2)
DZG16	roof tile	51° 32.517'N, 09° 56.175'E	38	18					1		fungi (19)
GWW	building facade	51° 32.422'N, 09° 56.502'E	22	19	1	2					
MA	concrete wall, shaded	51° 32.307'N, 09° 56.222'E	33		1	1	1			10	*Klebsormidium* (17), *Trentepohlia* (1), fungi (2)

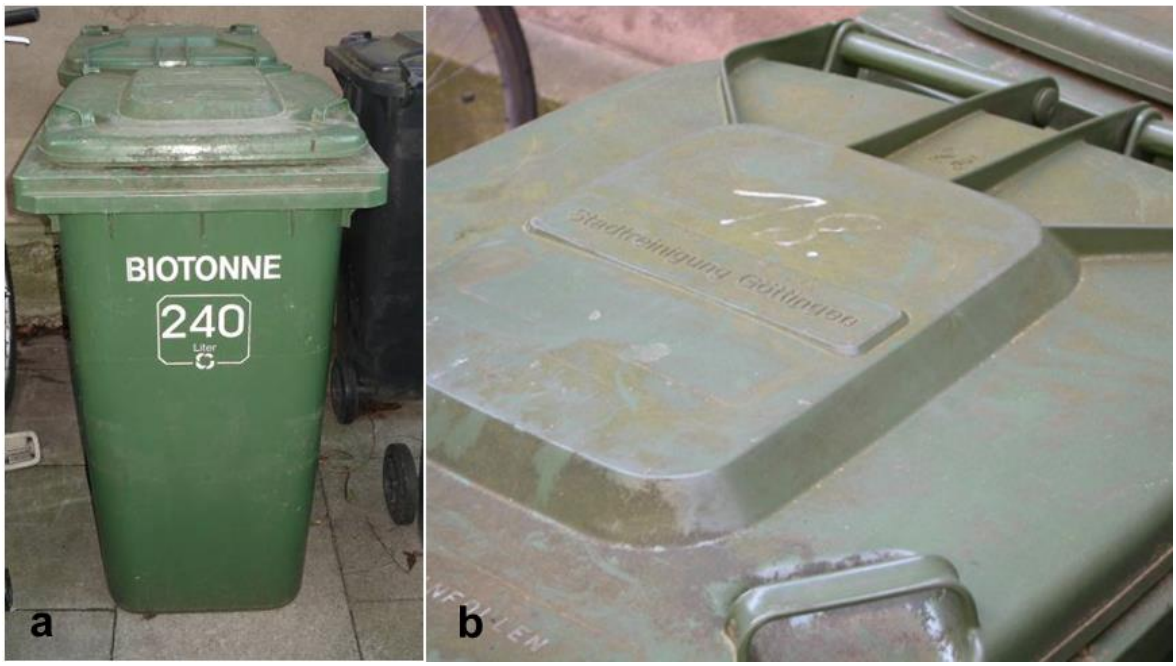

Fig. 1. Study site of an air-exposed phototrophic green biofilm (exemplar site GOG). (a) polyethylene compost waste container (b) biofilm on the surface of the waste container lid.

DNA extraction, PCR amplification, cloning and sequencing.

Genomic DNA was extracted from the biofilm samples, the GOG liquid crude culture and isolates. Two different methods were used for cell disruption before DNA preparation. For the cell disruption by shaking in a Mini-BeadBeater (Biospec, Barlesville, OK, USA) samples were divided into two fractions for different bead beating times. Equivalent amounts of 120-200 µm and 425-600 µm diameter acid washed glass beads (Sigma-Aldrich, ST. Louis, MO, USA) were added to 2 ml reaction tubes containing the samples which were vortexed briefly. The samples were treated in the bead beater once for 30 s and once for 50 s at 5.000 rpm. The biofilm samples were also disrupted by repeated freeze-thawing, i.e. samples frozen in liquid nitrogen were ground with a micropestle in 1.5 ml reaction tubes placed in aluminium blocks, pre-cooled in liquid nitrogen and then thawn. DNA was extracted with the Invisorb® Spin Plant Mini Kit (Stratec Molecular, Berlin, Germany), following the manufacturer's instructions. Quality of the DNA after extraction was checked on a 1 % (w/v) agarose gel. Isolated DNA was stored at -20 °C until further processing.

Eukaryote specific primer pairs NS1 (White et al., 1990) and 18L (Hamby et al., 1988) and NS1/LR1850 (Friedl, 1996) were used for the amplification of rRNA genes. The primer set NS1/18L amplifies the 18S rRNA gene region only, approximately 1800 bp

length, used for the biofilm sample. The primer set NS1/LR1850 amplifies the 18S rRNA gene, internal transcribed spacer 1 (ITS1), 5.8S rRNA gene, ITS2 and partial 26S rRNA gene regions, approximately 2300 bp length, used for biofilm sample, crude culture and isolates. About 10-100 ng of the extracted DNA were used as template. The amplification reaction mixture (50 µl) contained each dNTP at a concentration of 0.1 mM, 5 µl of 10 x reaction buffer, 2 mM $MgCl_2$, each primer at a concentration of 0.2 µM, 2 U of Taq DNA polymerase (Bioline, Luckenwalde, Germany) and 4 % (v/v) dimethyl sulfoxide (DMSO)-solution. PCR was performed in a PTC 200 thermocycler (MJ Research, Waltham, MA, USA) using the following program for the primer set NS1/18L: initial denaturation at 95 °C for 5 min, followed by 33 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 45 s, extension at 72 °C for 3 min and final extension at 72 °C for 7 min. For the primer set NS1/LR1850, the following program was used: initial denaturation at 95 °C for 5 min, followed by 33 cycles of denaturation at 94 °C for 40 s, annealing at 52 °C for 90 s, extension at 72 °C for 90 s, followed by 6 cycles of denaturation at 94 °C and final extension at 72 °C for 2 min. The PCR products were purified using the Invisorb^R Spin PCRapid Kit (Stratec Molecular). Aliquots of 2 µl of purified amplicons were analysed by electrophoresis on a 1 % (w/v) agarose gel.

Cloning was carried out with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) as described in Hallmann et al. 2013a. About 50 positive clones were cultivated overnight in LidBac reaction tubes (Qiagen, Hilden, Germany) with 1 ml LB medium containing 100 µg ampicillin. Plasmid DNA was purified with the NucleoSpin-Plasmid kit (Macherey and Nagel, Düren, Germany) and stored at -20 °C. Sequencing reactions were conducted with the Dye Terminator Cycle Sequencing v3.1 kit (Applied Biosystems, Darmstadt, Germany) and an ABI Prism 3100 (Applied Biosystems) automated sequencer. All clones were sequenced with the 18S rRNA gene standard sequencing primers either 895R (Hallmann et al., 2013a) or 34F (5' GTCTCAAAGATTAAGCCATGC 3') which yielded partial sequences of about 700 nts and included the hypervariable regions V2-V4. The sequences were edited using SeqAssem (Hepperle, 2004).

DNA sequence and phylogenetic analyses.

Sequences shorter than 420 bp without introns were excluded from further analysis. The sequences were compared with similar sequences of reference organisms by performing a BLASTn search at NCBI (Altschul, 1990; http://www.ncbi.nlm.nih.gov/). About 550 next relative sequences were imported into the ARB program (Ludwig et al., 2004;

www.arb-home.de), additional to internal sequence references. To determine the first phylogenetic affiliation the partial sequences were aligned with the homologous eukaryotic 18S rRNA gene sequences by using the automatic alignment tool of the ARB program package. Potential chimeras were checked by Bellerophon (Huber et al., 2004), the first and the last 300 bp of the sequence were compared with similar rRNA gene sequences in NCBI and putative chimeras were excluded from the dataset. Rarefaction curves and OTUs with ≥ 99 % similarity were calculated with MOTHUR (Schloss et al., 2009).

One up to four sequences of each OTU were selected and sequenced completely with standard sequencing primers. From isolates full length of the18S rRNA gene and the ITS region were sequenced. The sequences obtained in this study and internal sequence references were deposited in GenBank under the following accession numbers: JX169825 - JX169846 and KM116459 - KM116466. The alignment was performed using MAFFT version 6 (Katoh and Toh, 2008), small corrections were done by eye, using BioEdit alignment editor (Hall, 1999). The alignment included 109 sequences and 1794 positions (677 variable/485 parsimony informative sites).

Phylogenies were constructed by using the maximum likelihood (ML) method by RAxML (Stamatakis et al., 2008), using the GTR+Γ+I model with 100 replicates and with Bayesian methods (Huelsenbeck and Ronquist, 2001). Four Markov chains and three million generations sampling every 100 generations were applied, using the GTR+Γ+I model. Phylogenetic trees were viewed with FIGTREE (http://tree.bio.ed.ac.uk/software/figtree/).

Pairwise sequence similarities calculated from p-distances for representative clone and isolate sequences in comparison with reference sequences were computed with the program MEGA6 (Tamura et al., 2013).

Microscopy

For fluorescence light microscopy, the cells were double-stained with concanavalin A, coupled to fluorescein isothiocyanate (FITC; Sigma-Aldrich). The dye was applied in 50 mM potassium phosphate buffer, supplemented with 0.01 mM magnesium chloride and 0.01 mM calcium chloride at a dilution of 1/1000 of the original stock solution. The sample was inspected under a fluorescence light microscope (Axioscope, Zeiss, Göttingen, Germany; excitation wavelength 495 nm, emission wavelength 517 nm, Zeiss filter set 09). For staining of the EPS, samples on microscopy slides were covered with drops of indian ink (Pelikan, Hannover, Germany) or 1 % methylene blue (Merck, Darmstadt, Germany) prior to light microscopy.

For transmission electron microscopy (TEM), cells were harvested by short centrifugation at 10.000 rpm, resuspended in 50 mM potassium phosphate buffer, chemically fixed in 0.5 % (w/v) formaldehyde and 0.3 % (w/v) glutardialdehyde solution for 90 min at 0 °C, dehydrated in a graded methanol series and embedded in Spurr resin (Spurr, 1969; Hoppert and Holzenburg, 1998). Resin sections of 80-100 nm in thickness were cut with glass knives. Finally, the sections were stained with 0.5 % (w/v) phosphotungstic acid, pH 7.0, for three minutes. Electron microscopy was performed in a Zeiss EM 902 transmission electron microscope (Zeiss SMT, Oberkochen, Germany), equipped with a 1K digital camera, at 80 kV acceleration voltage and at calibrated magnifications.

Results

Biofilm clone libraries

The 18S rRNA gene clones from the studied green phototrophic biofilms revealed the dominance of *Apatococcus*. *Apatococcus* sequences were the only green algal sequences retrieved from the biofilms of four localities irrespective of the substratum (wood, EGU; metal, GLB, GLG, GLK; Table 1). Clones representing *Apatococcus* were dominant in the clone libraries established for three more localities with stone or plastic surfaces (DZG16, GOG, SKL). There, *Trebouxia* was the 2nd most abundant green alga and/or a third algal genus, *Coccomyxa* (DZG16), *Chloroidium* (GWW), *Pabia* (SKL) was retrieved by just one or two clones each. *Trebouxia* was equally dominant as *Apatococcus* in one sample (GSW) and at another locality (SK) *Trebouxia* was even the most dominant alga. The ten *Apatococcus* biofilms were at open and dry localities, i.e. they were not covered by vegetation and stayed humid only during and shortly after rainfalls. *Apatococcus* clones were absent in a clone library from a single locality which was less dry and sun-exposed than the other localities (MA, a humid concrete wall shaded by vegetation). At the latter locality 29 clones even represented green algae not retrieved from the other study sites (Table 1).

Microscopic observations of the ten *Apatococcus*-dominated biofilms always revealed green algal morphotypes besides the cell packages of *Apatococcus*, i.e. round or ovoid cells with a *Chlorella*- or *Coccomyxa*-like appearance and sometimes also round green algal cells covered by mucilage which may be assigned to Radiococcaceae following traditional green algal systematics (Ettl and Gärtner, 1995; Kostikov et al., 2002). However, algae

corresponding to these morphotypes were hardly retrieved from the clone libraries of the *Apatococcus*-dominated biofilms (Table 1). To test whether a larger algal diversity is present but simply cannot be detected adequately due to the dominance of *Apatococcus* and *Trebouxia* in these biofilms, we investigated crude cultures from a single locality using the same cloning/sequencing approach. It appeared that in the crude cultures growth of *Apatococcus* was somehow suppressed, i.e. it grew much slower than other terrestrial green algae.

Biofilm algae from cultures

We selected locality GOG because there the presence of *Chlorella*-, *Coccomyxa* and Radiococcaceae morphotypes was obvious in microscopy of the biofilms even without culturing. Microscopy of the GOG biofilm samples showed the dominance of *Apatococcus* cell packages and *Trebouxia* cells over inconspicuous, *Chlorella*-like or radiococcacean green algal cells (Fig. 2a). In addition to green algae, various filamentous fungi could be discerned (Fig. 2a), but no other algae or cyanobacteria. The crude cultures, i.e. after incubation of the biofilm material in liquid culture media for about four weeks, revealed a different picture. As expected, the *Apatococcus* cell packages were almost absent, but colonies of spherical cells with mucilage, *Pabia* morphotype, and long filaments of *Klebsormidium* had appeared, together with autospores packages-forming *Trebouxia* spp., numerous *Chlorella*- and *Coccomyxa*-like green algal cells (Fig. 2b). In addition, green algae of the *Chloroidium* morphotype were present.

The 18S rRNA gene clone library from the crude (enrichment) cultures revealed 83 green algal clones, but in contrast to the biofilm library no fungal clones were detected. We used a 99 % threshold to group the partial sequences obtained for the algal clones into OTUs. Rarefaction curves revealed a nearly full coverage of the crude culture library while almost a plateau for the biofilm clone library was found (Fig. 3). With both clone libraries a total of twelve green algal OTUs was detected and, as expected, both libraries were rather different in their OTU composition (Table 2). While just five green algal OTUs were recovered in the GOG biofilm library, nine were detected in the GOG crude culture library (Table 2). Interestingly, both libraries shared only two green algal OTUs, i.e. *Apatococcus* OTU 1 and *Trebouxia* OTU 10. *Chloroidium* OTU 8 was most dominant in the crude culture library (43 clones; Table 2).

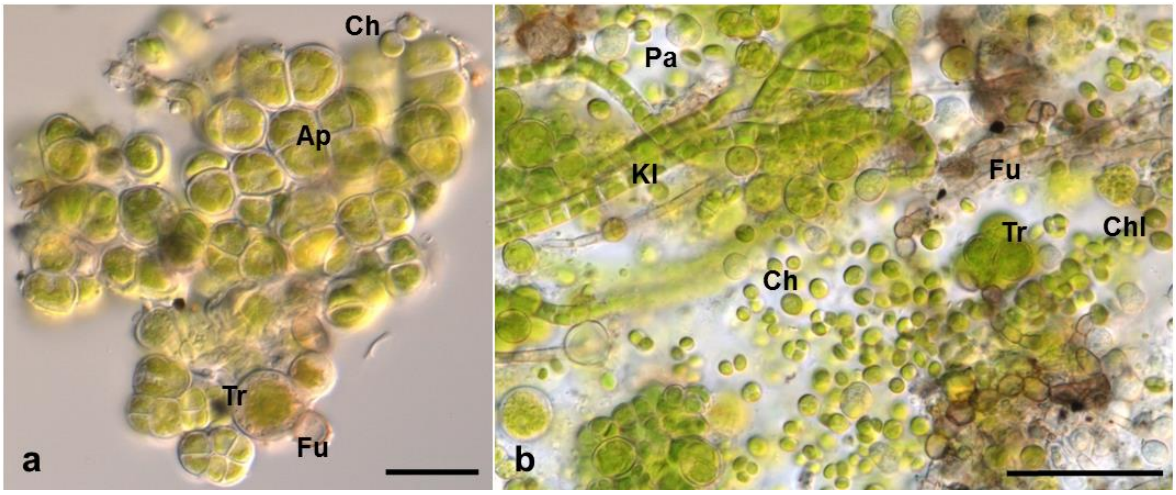

Fig. 2. Microscopy of the green biofilm at site GOG and crude culture developed from the same biofilm. (a) Biofilm sample with green algal morphotypes (Ap, *Apatococcus*; Ch, *Chlorella*-like; Tr, *Trebouxia*) and fungal hyphae (Fu). Scale bar, 20 µm (b) Crude culture with coccoid green algal morphotypes (Ch, *Chlorella*-like; Chl, *Chloroidium*; Pa, *Pabia*; Tr, *Trebouxia*), filaments of *Klebsormidium* (Kl) and fungi (Fu). Scale bar, 50 µm.

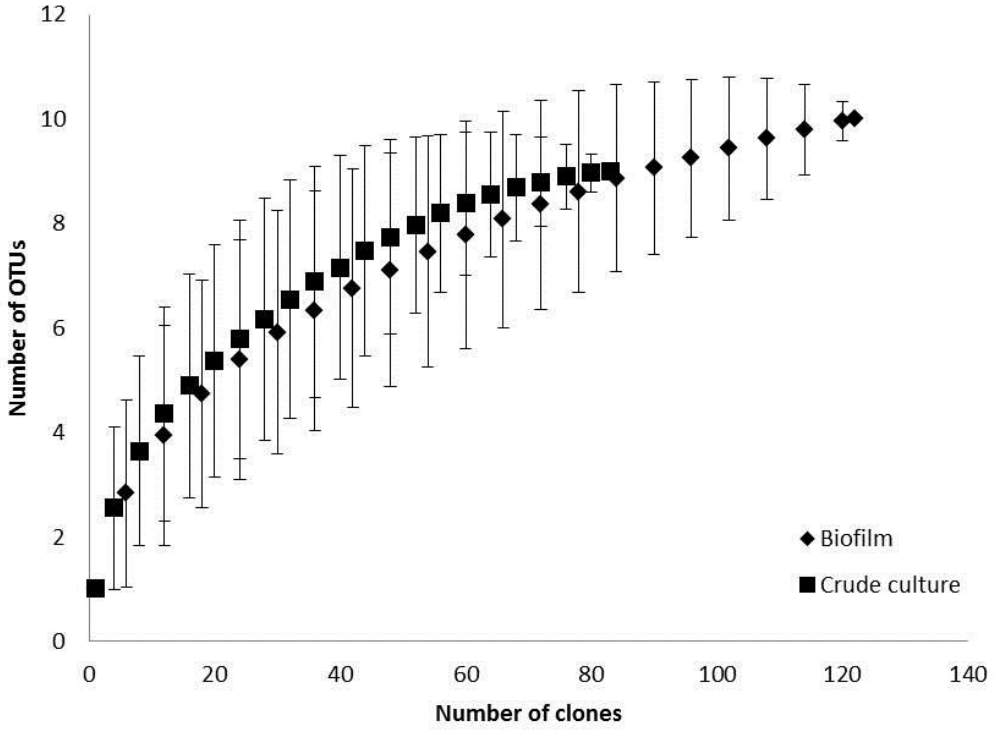

Fig. 3. Rarefaction curves for the 18S rRNA gene clone libraries established from the biofilm material and the crude culture of site GOG.

For a more reliable identification of the recovered green algae, for each OTU at least one almost full 18S rRNA gene sequence was determined. Using BLASTn the next closest neighboring available sequences (range of 99 % to 100 % from pairwise sequence similarities with the clones calculated from p-distances, see Table 2) were retrieved. In addition, five green algal strains, isolated from similar artificial substrates of urban environments as the GOG biofilm and available from the SAG culture collection, were sequenced and added to the data set of almost full 18S rRNA gene sequences (accession nos. KM116459 - KM116461, KM116463 and KM116465). From the latter data set of almost full 18S rRNA gene sequences OTUs (99 % sequence similarity threshold) were determined and a selection of sequences for each OTU was used for the phylogenetic analyses with a set of additional reference sequences (Table 2).

The biofilm algae were distributed on five distant groups (clades) of the Trebouxiophyceae (Fig. 4). For *Apatococcus* two OTUs were revealed which formed two clearly separated lineages in the 18S rRNA gene phylogeny; it is tempting to suspect both represent two distinct species (Fig. 4). OTU 1 included *A. lobatus* SAG 2037 which, because no authentic strain for the species is available, may be used as a reliable reference for the species. The strain has morphologically been well characterized as strain ASIB G177 in Gärtner and Ingolić (1989) and compared with the iconotype (Petersen, 1928). Strain SAG 2359 which we isolated from the GOG biofilm represented the same OTU and also exhibited the same morphology as *A. lobatus* SAG 2037 (Fig. 5a). Both strains shared 99.9 % pairwise sequence similarity with each other (Table 1), i.e. were different only in a single sequence position of the 18S rRNA gene region V4.

For *Apatococcus* OTU 2 four almost full 18S rRNA gene sequences were determined and they had rather high similarities (99.6 % to 100 %) with another reference strain, *Apatococcus* SAG 2151 (Table 2). For *Trebouxia* three OTUs were detected and they represented three independent lineages within the monophyletic clade representing the genus and, therefore, may represent three distinct species. OTU 5 was a close relative with the authentic strain of *T. impressa*, whereas OTUs 9 and 10 were closer related to the authentic strain of *T. asymmetrica* (Friedl and Gärtner, 1988) and a reference strain for *T. arboricola* (Gärtner, 1985).

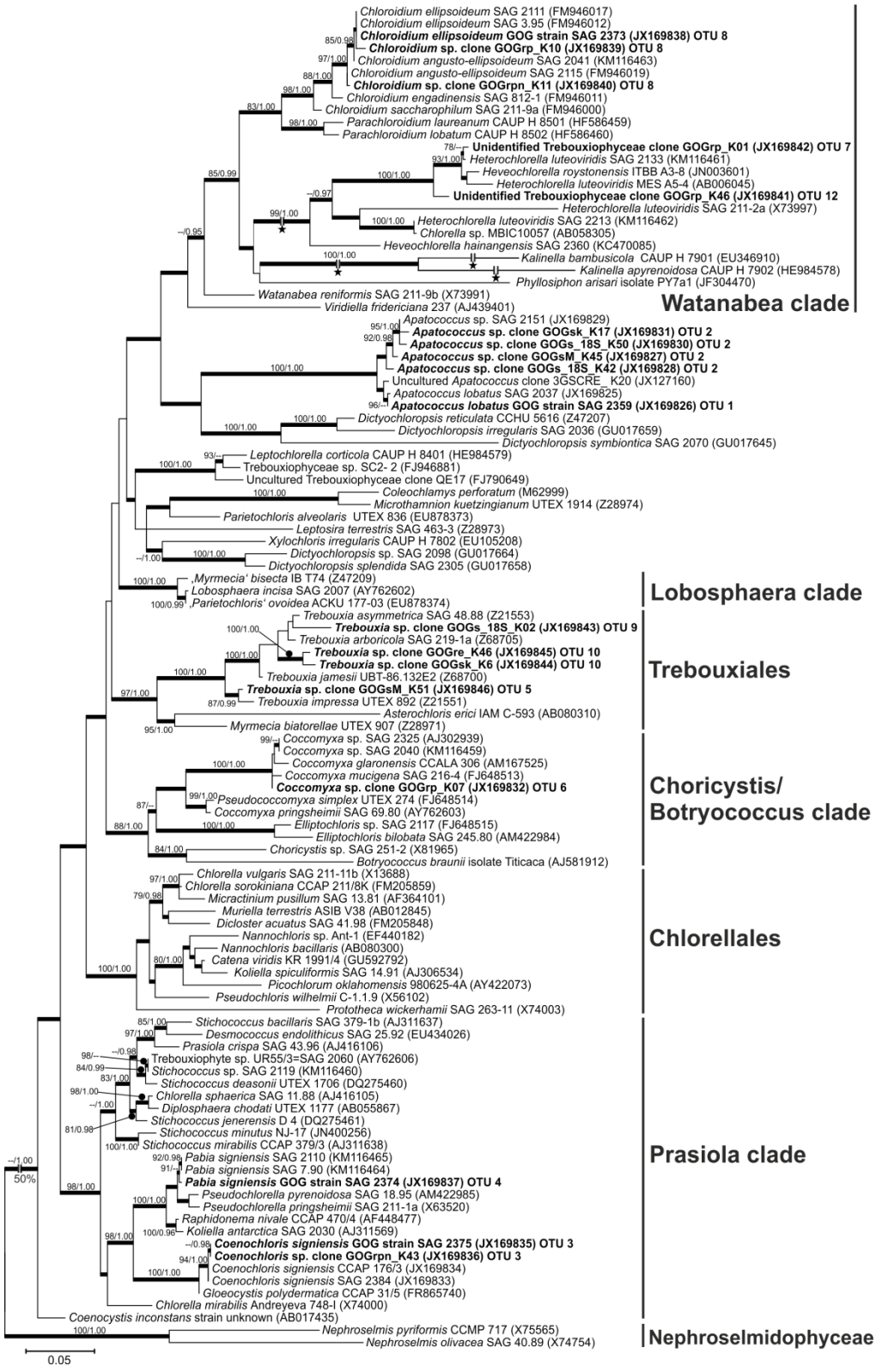

Fig. 4. Maximum-likelihood (ML) phylogeny of 18S rRNA gene sequences obtained from green biofilms using clone libraries and four isolates of the GOG exemplar site and other members of the Trebouxiophyceae, with members of Nephroselmidophyceae as outgroup taxa. Thick lines mark those internal branches which were received in both ML and Bayesian analyses (BI). Numbers at nodes correspond to ML bootstrap / BI posterior probabilities. Values only > 75 % (bootstrap) or > 95 % (posterior probabilities) are recorded. Asterisks mark those branches which were reduced to 50 % in lengths for the graphic.

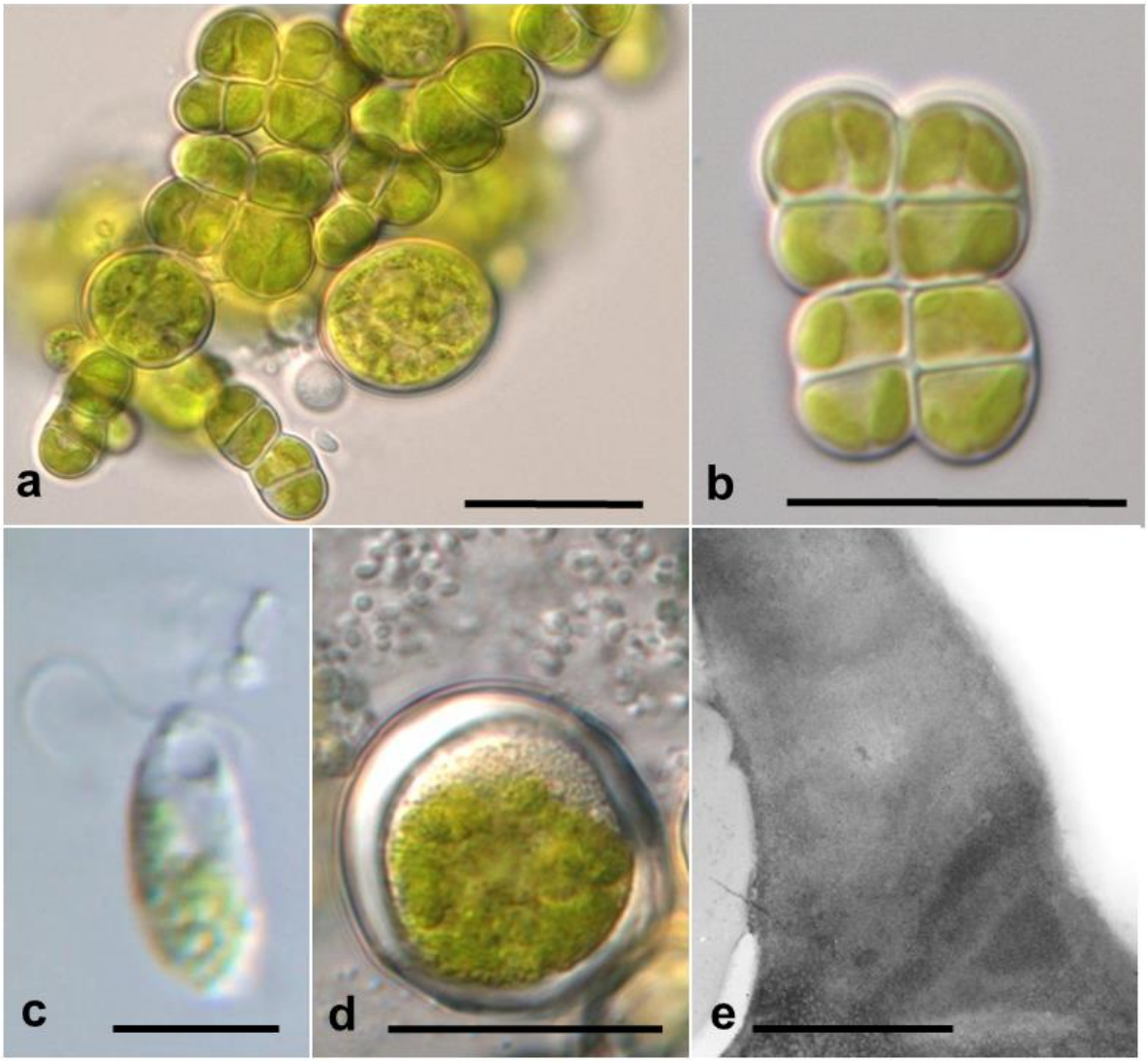

Fig. 5. Morphology of isolate *Apatococcus lobatus* SAG 2359 developed from the GOG study site. (a) Vegetative cells of various sizes. (b) Cell package. (c) Zoospore with two flagella and apical vacuole. (d) Cell with very thick wall of an old culture. (e) TEM detail of cell walls of two neighboring autospores. Scale bar, 0.5 µm (e) or 5 µm (c) or 20 µm (a, b, d).

Table 2. Green algal OTUs in the 18S rRNA gene clone libraries from the GOG biofilm, their genus assignment, number of retrieved clones per OTU, representing clone or isolated strain sequence for OTU and their percentage identities calculated from p-distances with closest available relative.

Genus	OTU no.	No. of clones biofilm/crude culture	Clone or isolate sequence	Percentage identity	Closest reference strain sequence and habitat
Apatococcus	OTU 1	19 / 2	*A. lobatus* strain SAG 2359 (JX169826)	99.9	*A. lobatus* strain SAG 2037 (JX169825); tree bark
	OTU 2	75 / 0	*A*. sp. clone GOGs_18S_K50 (JX169830)	100	*A*. sp. strain SAG 2151 (JX169829); plastic switchbox surface
			A. sp. clone GOGsk_K17 (JX169831)	99.8	
			A. sp. clone GOGsM_K45 (JX169827)	99.7	
			A. sp. clone GOGs_18S_K42 (JX169828)	99.6	
Trebouxia	OTU 5	5 / 0	*T*. sp. clone GOGsM_K51 (JX169846)	99.6	*T. impressa* strain UTEX 892 (Z21551); photobiont
	OTU 9	1 / 0	*T*. sp. clone GOGs_18S_K02 (JX169843)	99.2	*T. asymmetrica* strain SAG 48.88 (Z21553); photobiont
	OTU 10	2 / 2	*T*. sp. clone GOGre_K46 (JX169845)	99	*T. asymmetrica* strain SAG 48.88 (Z21553); photobiont
			T. sp. clone GOGsk_K6 (JX169844)	98.9	
Coccomyxa	OTU 6	0 / 2	*C*. sp. clone GOGrp_K07 (JX169832)	99.8	*C. mucigena* SAG 216-4 (FJ648513); photobiont
				99.8	*C.* sp. strain SAG 2325 (AJ302939); *Gingko biloba* endophyte
				99.8	*C*. sp. strain SAG 2040 (KM116459); roof tile
				99.6	*C. glaronensis* strain CCALA 306 (AM167525); *Gingko biloba* endophyte
Chloroidium	OTU 8	0 /43	*Chl. ellipsoideum* strain SAG 2373 (JX169838)	100	*Chl. ellipsoideum* strain SAG 3.95 (FM946012); unknown
				100	*Chl. ellipsoideum* strain SAG 2061 (FM946016); plastic switchbox
				100	*Chl. ellipsoideum* strain SAG 2111 (FM946017); roof tile
				100	*Chl. ellipsoideum* strain CAUP H1949 (FM946013); tree bark
				99.9	*Chl. ellipsoideum* strain SAG 2041 (KM116463); roof tile
			Chl. angusto-ellipsoideum clone GOGrpn_K11 (JX169840)	99.9	*Chl. angusto-ellipsoideum* strain SAG 2115 (JX169840); silicone surface
			Chl. sp. clone GOGrp_K10 (JX169839)	99.8	*Chl. ellipsoideum* strain SAG 3.95 (FM946012); unknown
unidentified Trebouxiophyceae	OTU 7	0 / 17	unidentified Treb. clone GOGrp_K01 (JX169842)	99.8	*Heterochlorella* sp. strain SAG 2133 (KM116461); roof tile
	OTU 12	0 / 2	unidentified Treb. clone GOGrp_K46 (JX169841)	98.8	*H*. sp. strain SAG 2133 (KM116461); roof tile
				99.5	*Heveochlorella roystonensis* strain ITBB A3-8 (JN003601); tree bark
				98.5	*H. roystonensis* strain ITBB A3-8 (JN003601); tree bark
Pabia	OTU 4	0 / 1	*P. signiensis* strain SAG 2374 (JX169837)	99.9	*P. signiensis* strain SAG 7.90 (KM116464); Antarctic soil
				99.9	*P. signiensis* strain SAG 2110 (KM116465); roof tile
Coenochloris	OTU 3	0 / 2	*Coe. signiensis* strain SAG 2375 (JX169835) and	99.9	*Coe. signiensis* strain CCAP 176/3 (JX169834); Antarctic soil
			clone GOGrpn_K43 (JX169836)	99.9	*Coe*. sp. strain SAG 2384 (JX169833); peatbog

Six more OTUs, assigned to at least five different genera of Trebouxiophyceae, were retrieved only from the crude cultures. OTU 6 *Coccomyxa* sp. was represented as a well-supported monophyletic lineage in the 18S rRNA gene phylogeny (Fig. 4). It included the authentic strain of *C. mucigena* SAG 216-4 which has been isolated from a lichen as well as several unidentified strains of *Coccomyxa* isolated from different terrestrial habitats, i.e. a roof tile (SAG 2040) and as an endophyte in *Gingko biloba* tissue (SAG 2325). Interestingly, the *Coccomyxa* was paraphyletic with *Pseudococcomyxa* within the *Choricystis/Botryococcus*-clade of the 18S rRNA gene phylogeny (Fig. 4).

Three more OTUs represented three independent lineages of the *Watanabea*-clade. OTU 8, represented by two clones and strain SAG 2373 isolated from the GOG biofilm, also included reference strains for species of *Chloroidium*, i.e. *Chl. ellipsoideum* SAG 3.95 (authentic strain) and *Chl. angusto-ellipsoidea* SAG 2115 (epitype strain; Darienko et al., 2010). Strain SAG 2373 exhibited 100 % 18S rRNA gene sequence identity with strain SAG 3.95 and also had only a single sequence position different in the ITS2 rRNA gene with the latter strain. Therefore, strain SAG 2373 was assigned to *Chl. ellipsoideum* (Table 2). Strain SAG 2373 exhibited also very high 18S and ITS2 rRNA gene sequence similarities (100 % and 99.9 %) with strains isolated from similar habitats, i.e. an air-exposed plastic surface in an urban environment (SAG 2061), a roof tile from a building in the close vicinity of GOG (SAG 2111), but also from tree bark (CAUP H 1949). There was even a 100 % ITS2 rRNA gene sequence identity with a strain isolated from a freshwater pond in Japan, SAG 2140. Clone GOGrpn_K11 (JX169840) from the crude culture library had 99.9 % 18S rRNA gene sequence similarity with the epitype strain of *C. angusto-ellipsoideum* SAG 2115 which was isolated from a similar urban habitat in the close vicinity of GOG, but 99.7 % with *Chl. ellipsoideum* SAG 3.95 and, therefore, was assigned to the former species. Clone K10 (JX169839) had genetic similarities with the *C. angusto-ellipsoideum* and *Chl. ellipsoideum* reference strains shorter than 99.9 % (99.5 % and 99.8 %) and, therefore, may represent an independent species of *Chloroidium*.

The crude culture clones representing OTU 7 and OTU 12 were within a well-supported monophyletic lineage within the *Watanabea*-clade of the 18S rRNA gene phylogeny which also included strains assigned to *Heterochlorella luteoviridis* and *Heveochlorella roystonensis* (Fig. 4). Because the authentic strain of *H. luteoviridis* SAG 211-2a was not included into this lineage and rather represented an independent lineage of the *Watanabea*-clade, the lineage may not represent *H. luteoviridis*. *Heterochlorella* is paraphyletic with *Heveochlorella* in the 18S rRNA gene phylogenies, i.e. the authentic

strain of the type of *Heveochlorella*, *H. hainangensis*, and the strain representing *H. roystonensis* did not form a monophyletic lineage either, but were separated from each other on rather distant lineages within the *Watanabea*-clade (Fig. 4). Therefore, no species and generic assignments are yet possible for OTU 7 and OTU 12 and we refer them to "unidentified Trebouxiophyceae" (Tables 1 and 2) here. Based on 18S rRNA gene genetic distances and phylogenetic relationships, their closest relatives were strains isolated from a roof tile, SAG 2133, and from tree bark, *H. roystonensis*, as well as a strain of unknown origin (MES A5-4).

OTU 3 and OTU 4 represented two independent lineages within the distant *Prasiola*-clade of Trebouxiophyceae (Fig. 4). They were related to species which form spherical cells with mucilage which in traditional green algal systematics were assigned to the family Radiococcaceae (Kostikov et al., 2002). Strain SAG 2374 (OTU 4) isolated from the study site exhibited this morphotype (Fig. 6a). It had a 18S rRNA gene sequence similarity of 99.9 %, corresponding to two positions different in the 18S rRNA gene V4 region, with strain *Pabia signiensis* SAG 7.90 which is the authentic strain of the type species of the genus (Friedl and O'Kelly, 2002) and, therefore, is assigned to this species. Interestingly, strain SAG 7.90 has been isolated from soil in Antarctica (isolated by PA Broady, 1979).

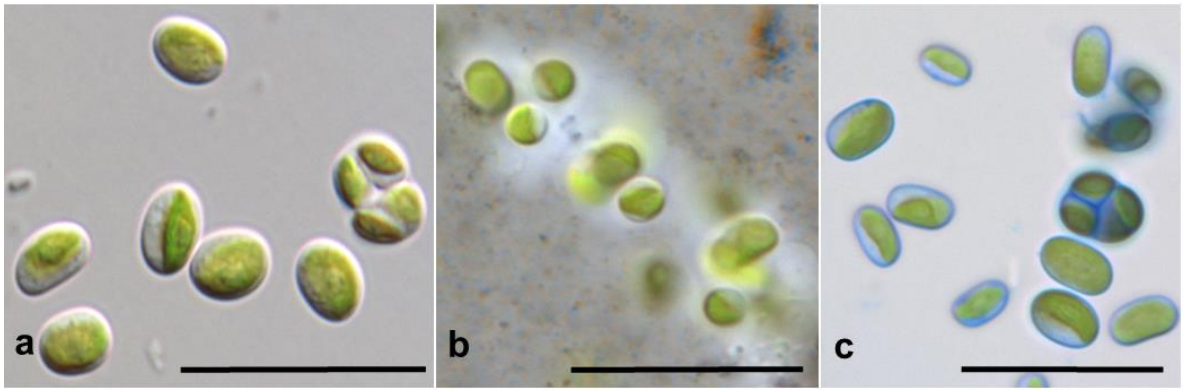

Fig. 6. Morphology of isolate *Pabia signiensis* SAG 2374 developed from the GOG study site. (a) Vegetative cells and autosporangia. (b) Mucilage after negative staining with indian ink. (c) Vegetative cells after staining with methylene blue. Scale bars, 20 µm.

Strain SAG 2374 had the same high 18S rRNA gene similarity with strain SAG 2110 which has been isolated from a roof tile of a building in the close vicinity of GOG. At the ITS2 rRNA gene level, strain SAG 2374 had eight and nine sequence positions different with strains SAG 7.90 and SAG 2210. OTU 3 was represented by two identical 18S rRNA gene sequences which were from an isolate from GOG, SAG 2375, and a crude culture clone (Table 2; Fig. 4). Both had 99.9 % pairwise 18S rRNA gene sequence similarity, corresponding to a single sequence difference in the V4 region with the authentic strain of the *Coenochloris signiensis* CCAP 176/3 and, therefore, the OTU was assigned to this species. Again, also the reference strain CCAP 176/3 has been isolated from Antarctica (by PA Broady, 1973; www.ccap.ac.uk). Strain SAG 2375 shared a 99.9 % sequence similarity with so far unidentified strain SAG 2384 and, therefore, also the latter strain is assigned to *Coe. signiensis* here. At the level of ITS2 rRNA gene sequences, there were 18 differences between strains SAG 2375 and SAG 2384. An environmental unidentified clone from a glacier forefield in the Alps (sequence JX435389) was with just 10 ITS2 sequence differences the closest relative with strain SAG 2375.

Microscopy of biofilm isolates

Unialgal cultures from the GOG biofilm could be established for four OTUs (Table 2). Strain *A. lobatus* SAG 2359 formed packages of thick-walled cells which were about 9-11 µm in diameter, autosporangia (cell packages) were up to 20 µm in size (Fig. 5a, b). The formation of zoospores in cultures of about two weeks old was also frequently observed, despite zoospore formation in *Apatococcus* has been reported only rarely so far (e.g. Gärtner and Ingolić, 1989). The zoospores were 6-7 µm in length, with two flagella and an apical vacuole, but without a stigma (Fig. 5c). Vegetative cells were covered by remnants of sporangial walls or were rather thick which was particularly frequently seen in old cultures (Fig. 5d). Transmission electron microscopy (TEM) depicted thick cell walls of about 1 µm (Fig. 5e).

Two strains represented the Radiococcaceae morphotype and were characterized by conspicuous formation of mucilage on agar plates, i.e. *P. signiensis* SAG 2374 (Fig. 6) and *Coe. signiensis* SAG 2375 (Fig. 7). SAG 2374 formed mostly ellipsoidal cells of 3-6 µm, chloroplasts containing a pyrenoid (Fig. 6a). The vegetative cells of SAG 2375 were ellipsoidal when young, later becoming spherical and were 3.5-12 µm in diameter (Fig. 7). The chloroplast was band-shaped with a pyrenoid (Fig. 7a). They formed 2-4 ellipsoidal autospores per cell, the cell division was oblique.

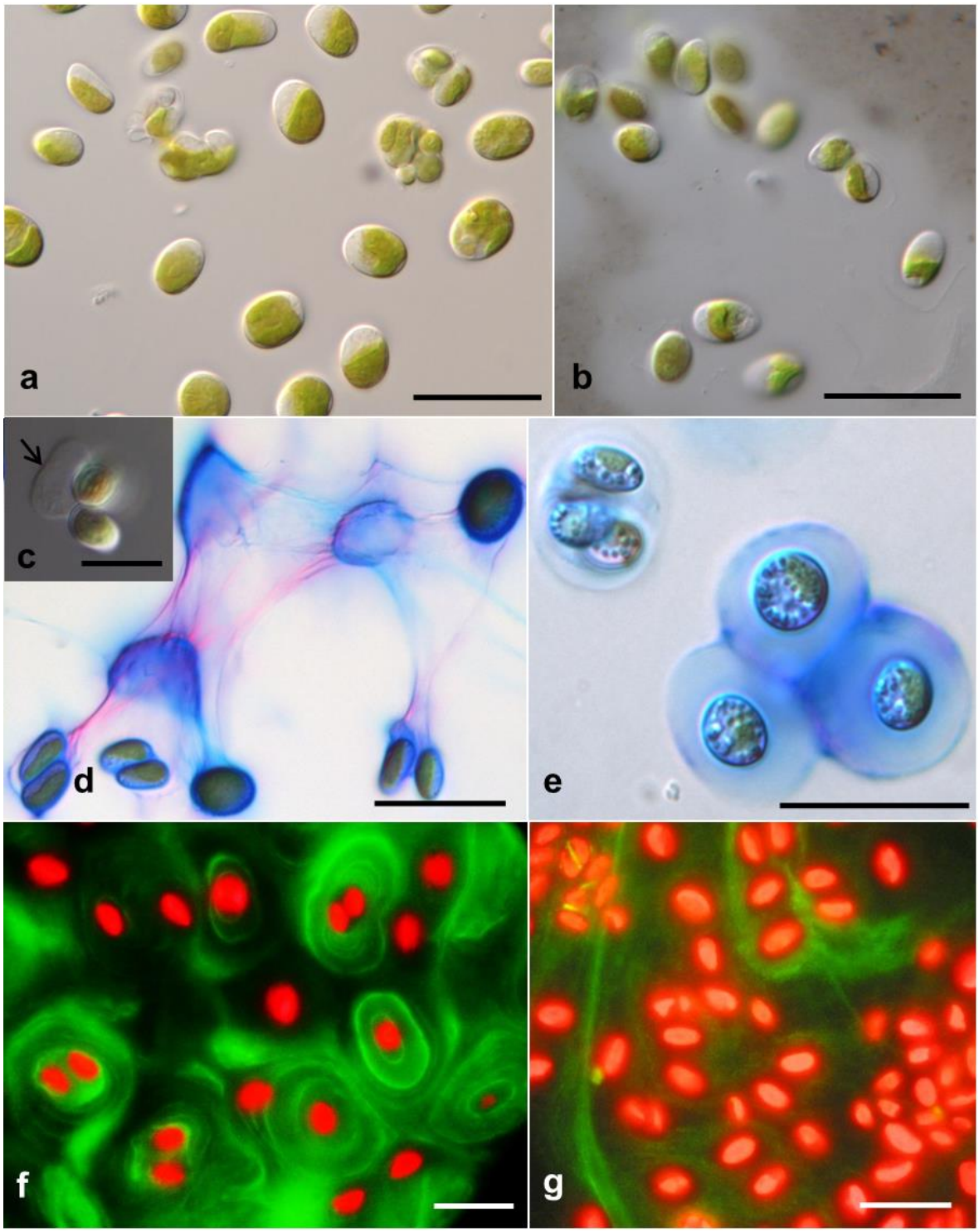

Fig. 7. Morphology of isolate *Coenochloris signiensis* SAG 2375 developed from the GOG study site. (a) Vegetative cells with smooth chloroplasts. (b) Mucilage after negative staining with indian ink. (c) Remnants of mother cell walls (arrow). (d) Vegetative cells with mucilage in young culture after staining with methylene blue. (e) Vegetative cells with mucilage in an old culture after staining with methylene blue. (f) Xenic culture, cells forming mucilage capsules. Green, Concanavalin A FITC conjugated stain; Red, autofluorescence of chlorophyll. (g) Axenic culture with loose mucilage. Green, Concanavalin A FITC conjugated stain; Red, autofluorescence of chlorophyll. Scale bar 5 µm (c) or 20 µm (a, b, d-g).

Remnants of sporangal walls sticked to vegetative cells were often observed (Fig. 7c). Both isolates produced exopolymer matrices on agar plates, although the *P. signiensis* SAG 2374 matrix appeared to be thinner and less rigid (Fig. 6b, c) than that of the *Coe. signiensis* SAG 2375 matrix (Fig. 7b-e). The latter could be identified as an exopolysaccharide (EPS), since Concanavalin A staining was positive (Fig. 7f, g). By this way, also concentric layers of different fluorescent intensities could be observed in older and xenic cultures (Fig. 7g), which are also typical for rigid exopolymer matrices. Interestingly, this feature could not be observed in axenic culture (Fig. 7f). Strain *Chl. ellipsoideum* SAG 2373 showed ellipsoidal cells, 4-9 µm in diameter, with lobed chloroplasts containing a pyrenoid (Fig. 8). The strain exhibited no special cell wall features as depicted by TEM (Fig. 8b, c).

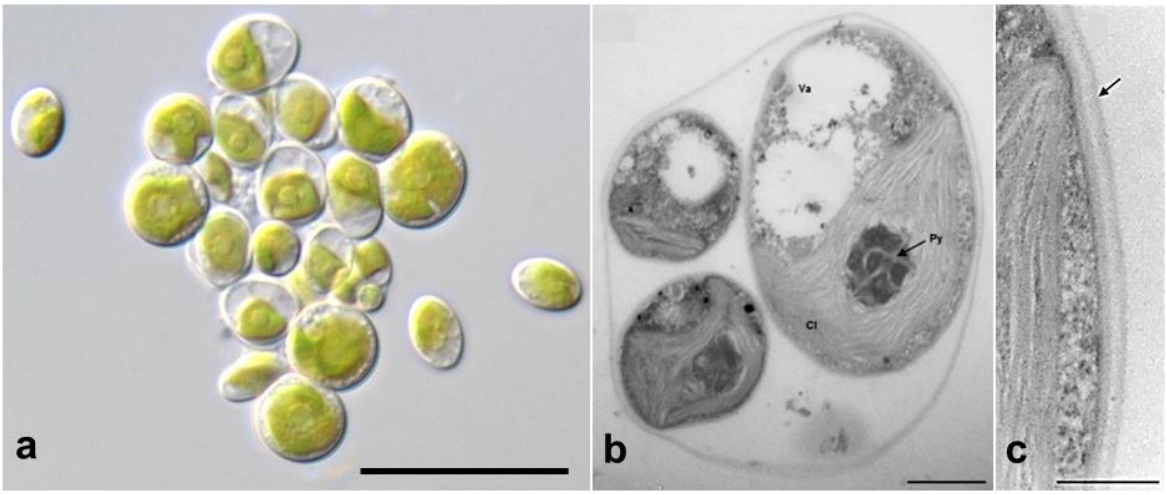

Fig. 8. Morphology of isolate *Chloroidium ellipsoideum* SAG 2373 developed from the GOG study site. (a) Vegetative cells and autosporangia. (b) TEM section of autosporangium. Cl, chloroplast; Py, pyrenoid; Va, vacuole. (c) TEM detail of thin cell wall of the autosporangium (arrow), close up of image (b). Scale bar, 0.5 µm (b, c) or 20 µm (a).

Discussion

Our study focused on eleven sites providing air-exposed surfaces of various artificial substrates for green phototrophic biofilms, ten sites were open dry air- and sun exposed higher than 1.5 m above the ground. These sites represent a particular type of green algal dominated biofilms, common in urban areas of Middle Europe, allowing a case study in order to assess the algal diversity of these biofilms, despite the number of investigated sites was still small and the study sites were within short distances of a single mid-sized town. All green biofilms were dominated by cell packages-forming green algae, which in previous works have been assigned to "*Apatococcus* and/or *Desmococcus*" or the *Pleurococcetum* community of aeroterrestrial green algae (e.g. Rindi, 2007). Our study using 18S rRNA gene clone libraries established from the dry biofilms without culturing clearly showed the biofilms being dominated by *Apatococcus* with *Trebouxia* as the second most abundant green algae, whereas *Desmococcus* was not detected. Although difficult to distinguish by microscopy (e.g. Gärtner, 1994; Lüttge and Büdel, 2010; Rindi, 2011), both genera are phylogenetically clearly separated from each other within the Trebouxiophyceae. *Apatococcus* forms a lineage by its own, i.e. there is no support for a closer relationship with another lineage of Trebouxiophyceae, although in some analyses a sister-group relationship with species of *Dictyochloropsis* and even the *Watanabea*-clade has been resolved, but without support in significance tests (Fig. 4; Hallmann et al., 2013a, 2013b; Neustupa et al., 2013). The analyses here substantiate this phylogenetic position with eight almost full 18S rRNA gene sequences from *Apatococcus* strains/clones of various origins. In contrast, strains of *Desmococcus* assume a position within the *Prasiola*-clade (Fig. 4; Mikhailyuk et al., 2008; Hallmann et al., 2013a).

The dominating *Apatococcus* in the dry sun-exposed biofilms is not just a single species, *A. lobatus* (Gärtner and Ingolić, 1989), but may consist of several species of *Apatococcus* which can be separated by the 18S and ITS2 rRNA gene sequence analyses. Two OTUs representing two distinct species of *Apatococcus* were retrieved from the GOG site with the biofilm clone libraries and they form two distinct lineages in the 18S rRNA gene phylogenies (Fig. 4). Partial 18S rRNA gene sequences identical to those from both *Apatococcus* OTUs at GOG were retrieved from five other studied sites (DZG16, SKL, GSW, GLG and GLB), three of the sites (DZG16, GLG and GLB) exhibited even another additional type of *Apatococcus* partial sequence. Only from three sites (EGU, GWW and SK) just a single *Apatococcus* sequence type, corresponding neither to OTU 1 nor OTU 2

were retrieved. The two OTUs of *Apatococcus* discriminated in the biofilm study here correspond to an earlier finding (Hallmann et al., 2013b).

Similarly, also three OTUs or species of *Trebouxia* were recovered from the GOG biofilm clone libraries (Table 1) and, as far as the sequence comparisons with the reference sequences for these OTUs (Table 2) allows at least two of them were found at two other study sites as well, SK and GWS. For identification of the *Trebouxia* OTUs to species level the 18S rRNA gene sequence comparisons may, however, not be sufficient and ITS rRNA gene sequences be required, also because there is a large number of ITS rRNA gene sequences for the various species of *Trebouxia* available (e.g. Ruprecht et al., 2014). Apart from species of *Apatococcoccus* and *Trebouxia* various filamentous fungi were recovered from the biofilm clone libraries of the sun-exposed sites in relatively high abundances. These fungi may live embedded together with the green algae in an exopolymeric matrix produced by the biofilm organisms.

Several fungi detected here may occur as lichen mycobionts, e.g. close relatives of *Heterodea muelleri* were retrieved (Wedin et al., 2000). Therefore, young inconspicuous initial stages of lichens or small lichen propagules consisting of both symbionts may have introduced the *Trebouxia* species (OTUs) into the biofilms although no detectable lichen thalli were found at the sampling sites. The *Trebouxia* cells in the biofilms might not have necessarily been free-living (Ahmadjian, 1988). *Trebouxia* has already been frequently described in terrestrial habitats, even when identifiable lichens were absent (e.g. Gärtner, 1994; Macedo et al., 2009). Also other microfungi not known as lichen-forming were detected, i.e. the *Hyphozyma*-related clones may represent free-living yeast-like hyphomycetes (De Hoog and Smith, 1986). The GOG biofilm clone library revealed a high abundance of the ascomycete *Glyphium elatum* which is known to develop peculiar black ascomata on tree bark, but has also been reported from chemically treated marble surfaces (Cappitelli et al., 2007). Although the biodegradability of high density polyethylene (HDPE), the substrate at the GOG site, by molds has been reported (Albertsson et al., 1978), it is more likely that the *Glyphium*-related fungi detected in the green biofilms use the green algae as a more convenient substrate. The relatively high number of retrieved *Glyphium* clones also makes it rather unlikely that our sample taken from GOG was just contaminated by allochthonous *Glyphium* mycelia.

The presence of just a few organisms but in relatively high abundances as observed for the studied biofilms may indicate an extreme habitat with life conditions favorable to only a small selection of algae. Also, the substrate surfaces at all our study sites have been

exposed for a long time, i.e. since several years, to the environment and, therefore, the biofilm may not consist of just transient "pioneer" colonizers. For example, the substrate of our exemplar study site, GOG, represents a chemically inert material, i.e. high density polyethylene (HDPE), which is rather hydrophobic, with a slightly negative water contact angle (Vogler, 1998). HDPE is a very abundant plastic material out of which numerous different products such as bins, tubes, signboards or street furniture which all can frequently be covered by green phototrophic biofilms are manufactured. HDPE provides a smooth, inert surface, which may be less favorable for colonization than natural surfaces such as stone or tree bark. Water on dry plastic material is less available since it does not penetrate the surface via small pores and ducts like on tree bark or natural stone. Although liquid water after rainfall provides a thin water film which may cover the biofilm on a horizontal face for even a longer time period than on a porous surface, no moisture from underlying material will be available to the biofilm. HDPE shows a low biodegradability (Fontanella et al., 2010) presenting a "neutral" substrate to adhering organisms without providing minerals and trace ions but also being non-toxic.

In concordance with regarding the exemplar site GOG as well as the other studied sites as extreme habitats is that two species, *Coenochloris signiensis* and *Pabia signiensis*, so far reported from the very harsh climate of Antarctica, have been found as colonizers of the sun-exposed dry biofilms (Tables 1, 2).

Our isolates established from the exemplar site GOG may exhibit some features which may be regarded as particular adaptation to their extreme habitat. The formation of thick cell walls of *Apatococcus* as seen by microscopy of the studied biofilms or in old cultures (e.g. the isolate from GOG, SAG 2359) may serve as an adaption to desiccation concurrent with high temperature amplitudes and UV-irradiation (Karsten et al., 2005a).

The isolates of *Coenochloris signiensis* and *Pabia signiensis* from GOG, SAG 2375 and SAG 2374, produced exopolymer matrices that appear to be functional in rather hydrated conditions on agar plates and in liquid cultures. However, no indication for a protective function of the EPS could be found upon drying and UV-irradiation, a feature that has been described for typical aeroterrestrial algae and cyanobacteria with rigid capsules (Wynn-Williams et al., 1997; Kemmling et al., 2004). In an aqueous habitat (or on a fully hydrated substratum like agar), the EPS may act as an adhesive between cells and mediate the attachment to the substrate. Induction of exopolysaccharide formation in bacterial biofilms has been observed frequently, e.g. by quorum sensing (e.g. Sakuragi and Kolter, 2007). For eukaryotes, the phenomenon has so far mostly been described only for

the interaction between symbiotic nitrogen-fixing bacteria and higher plants (e.g. Marketon et al., 2003), but one example for the secretion of EPS upon co-culturing with distinct *E. coli* strains has been described for the diatom *Phaeodactylum tricornutum* (Bruckner et al., 2011). *Chloroidium* isolate SAG 2373 had no visible adaptive structures to extreme environments, i.e. neither thick cell walls nor mucilage was observed. However, an unusual constitutive carbohydrate composition including the presence of polyols, e.g. ribitol, has been reported for *Chloroidium* strains and considered as effective stress metabolites (Darienko et al., 2010; Gustavs et al., 2011).

Six OTUs or species from at least five genera of Trebouxiophyceae were recovered exclusively in the clone library developed from crude cultures of the biofilm from the exemplar site GOG (Table 2). Though it may be expected that microbial diversity changes upon culturing, this feature has hitherto rarely been described for algae (Guillou et al., 2004; Miller et al., 2008; Hallmann et al., 2011). Various green algae, also those not from air-exposed habitats, can form drought-resistant dormant stages (Lennon and Jones, 2011) and could have been detected by our culture approach. However, except for *Coenochloris signiensis*, representatives of the other four genera were also retrieved from the biofilm libraries without culturing (Table 1). This together with the fact that all species detected in the crude culture clone library are common species of air-exposed habitats evidences that they do not represent just a small "seed bank" (Lennon and Jones, 2011; Hallmann et al., 2013a) of diverse algae which were just drifted onto the biofilm surfaces without actually being active members within the biofilms. In addition, four of the species found in crude culture have their closest relatives with isolates from habitats similar to the study sites (SAG 2040, SAG 2041, SAG 2110, SAG 2115 and SAG 2133; Table 2, Fig. 4). That these species were absent or mostly found with a single clone in the biofilm libraries without culturing may be simply due to that the *Apatococcus/Trebouxia* DNA was so abundant in the biofilm extractions that DNA of almost no other algae could become amplified. We experienced that establishing cultures of *Apatococcus* using standard methods was less successful than for most other terrestrial green algae and, therefore, the development of *Apatococcus* may have been suppressed in the liquid crude cultures, at the same time favoring the development of the other green algae already present in the biofilm. Also, the number of sequenced clones per biofilm library (21-102; Table 1) may still have been not sufficient to detect other green algae although the rarefaction analysis of the GOG biofilm clones indicated almost full coverage of the library (Fig. 3). Fungi could not be retrieved

from liquid crude cultures. Though also oligotrophic fungi may be present as saprophytes in algal cultures, liquid cultures are less favorable for fungal growth.

Probably due to constraints of the extreme habitat the biofilm green algae exhibited a high degree of morphological uniformity which, however, belied a variety of distinct genotypes or species which cannot be discerned by microscopy of the biofilms. Similarly to *Apatococcus* and *Trebouxia*, our sequence analyses of the crude cultures from exemplar site GOG revealed at least two species, *C. ellipsoideum* and *C. angusto-ellipsoideum*, co-occuring in the same biofilm. Species of *Chloroidium* are rather common in aeroterrestrial habitats (Darienko et al., 2010) with previously being reported from light microscopic studies of biofilms as a single species, "*Chlorella trebouxioides*" (e.g. Lüttge and Büdel, 2010).

The unidentified trebouxiophyte of the *Watanabea*-clade (previously assigned to *Heterochlorella*, see below) from GOG was present in two lineages (OTUs) and also our isolates of *Coenochloris signiensis* and *Pabia signiensis* from GOG were different at the rRNA gene level from previous isolates of the same species (Table 2). This "cryptic" (genetic) diversity may also be an adaptive feature of the algal community to the extreme biofilm habitat. Finally, the finding of genetic diversity in both the latter species is not in concordance with the classical tenet which says "Everything is everywhere, but, the environment selects" (Baas Becking, 1934; De Wit and Bouvier, 2006), but congruent with a similar finding of the same terrestrial microalgae species being distributed in Antarctica and temperate regions, but present as different "subpopulations" which previously has been reported for the Xanthophyceae (Stramenopiles, Rybalka et al., 2009, 2013).

Despite the biodiversity of green algae in the studied biofilms may have been low, i.e. no more than 11 OTUs or species of Trebouxiophyceae (Chlorophyta) were revealed in our study, also species or even genera so far not represented in molecular-phylogenetic analyses or even representing so far undescribed new taxa were found. OTUs 7 and 12 may represent two distinct species of a still undescribed genus, OTU 12 even a species at least so far not recovered in molecular phylogenetic analyses. OTU 7 may correspond to *Heveochlorella roystonensis*, together with other strains isolated from terrestrial habitats previously assigned to *H. luteoviridis*, because of their very close relatedness in the 18S rRNA gene phylogenies (Fig. 4; Table 2). However, *H. roystonenis* is not only distantly related within the *Watanabea*-clade to the type of *Heterochlorella*, *Het. luteoviridis* with SAG 211-2a as authentic strain, but also to the type of *Heveochlorella* which is *H. hainangensis* (Fig. 4; Zhang et al., 2008). The species needs to be transferred to another

genus. The finding of very close relatives of *H. roystonenis* in the exemplar GOG biofilm again supports a world-wide distribution of terrestrial green algae (Hodač et al., 2012); *H. roystonenis* has been reported only from tropical China so far (Ma et al., 2013).

The still unidentified species of *Coccomyxa* from the biofilms with no named closer related strain available yet, represented by our OTU 10 and two isolates from similar habitats, strains SAG 2040 and SAG 2325, was found as a close relative with lichen photobionts in the 18S rRNA gene phylogenies. *Coccomyxa* in its present circumscription was revealed not as a monophyletic lineage; rather it was separated into two phylogenetically distinct but related monophyletic lineages (Fig. 4). Zoller and Lutzoni (2002) showed that there are two distinct lineages of *Coccomyxa*, one including lichen photobionts and the other with only free-living non-symbiotic species. It is also likely that *Coccomyxa* OTU 10 was introduced by lichen propagules or initial stages, similar to the species of *Trebouxia* recovered from the studied biofilms.

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Chapter 5:
Green algal communities from soil and tree bark in middle European forests: a molecular comparison

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Manuscript

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Conceived and designed the experiments: TF, CH, LH.

Abstract

The composition of green algal communities from soils and tree barks was determined using 18S rRNA gene sequence comparisons. The samples were collected from nine defined research plots of the German Biodiversity Exploratory Schwäbische Alb, representing three different management types of spruce and beech forests. Following a culture-independent approach (cloning and sequencing) which was complemented by culturing of green algae, a total of 62 operational taxonomic units (OTUs) were identified. In soil 37 OTUs representing the green algal class Trebouxiophyceae and 15 OTUs affiliated to Chlorophyceae were detected. Most abundant OTUs in soil belong to *Desmococcus*, *Elliptochloris* and a group of so far unidentified *Prototheca*-relatives, which were retrieved exclusively through cloning. On tree bark the algal diversity was lower with 26 OTUs representing only Trebouxiophyceae, out of which *Apatococcus*, *Coccomyxa* and *Elliptochloris* were the most frequent ones. The microhabitats in forest soils are more heterogeneous than tree bark surfaces which is congruent with the different molecular diversities in soil and on tree bark we found within the same plots.

Key words: green algae, soil, forest, tree bark, 18S rRNA gene, OTUs, culture-independent.

Introduction

Terrestrial green algae are important primary producers in soils and on tree bark and were suggested as potential bioindicators (Bérard et al., 2005; Freystein et al., 2008). Large part of microbial diversity resides in forest soils and epiphytic on tree bark (e.g. Will-Wolf et al., 2002; O'Brien et al., 2005; Roesch et al., 2007). However, the ecological relevance of microbial diversity in these terrestrial ecosystems is poorly understood, in particular for eukaryotic organisms. Though the importance of fungi with respect to the remediation of organic biomass in soil is well recognized and epiphytic fungi and lichens are well known as abundant cryptogams on tree bark (Barkman, 1958; Elbert et al., 2012), the ecological role of algae has not been addressed so far in temperate ecosystems. It is known that microalgae, besides cyanobacteria, lichens and mosses, are important primary producers in Antarctic terrestrial ecosystems (Bölter, 1992; Broady, 1996; Feng et al., 2010 and references therein). In arid and semi arid habitats, microbiotic soil crusts, dominated by eukaryotic algae and cyanobacteria, are highly important for soil fertilization and mechanical stability (Lewis and Lewis, 2005; Büdel et al., 2009; Řeháková et al., 2011). In temperate soil ecosystems, the role of algae may be less pronounced. Since vascular plants are the predominant primary producers in soil ecosystems, a major contribution of algae to soil organic matter may not be expected. However, their importance as producers of organic matter were seemingly underestimated, which serves as nutrient source for microorganisms and invertebrates and were consumed by roots of plants (Kabirov and Gaisina, 2009).

Green algae (Chlorophyta) comprise the most part of the algal diversity in soils (Metting, 1981; Hoffmann, 1989). They improve soil fertility and trap particles together with the other groups of eukaryotic algae known to be abundant from soils besides chlorophytes, e.g. the heterokont algae, xanthophytes, eustigmatophytes and diatoms, improve soil texture and nitrogen-fixing cyanobacteria (Shields and Durrell, 1964; Metting, 1981; Hoffmann, 1989; García-Pichel et al., 2001). Diversity assessments of microalgal communities from temperate forest soil and the comparison of forest soil algal communities of different localities have so far been achieved only by morphology (Lukešová and Hoffmann, 1996; Hoffmann et al., 2007; Novakovskaya and Patova, 2007, 2008; Khaybullina et al., 2010). Earlier studies revealed first top 5 cm soils rather diverse in microalgae and that the microalgal communities may be sensitive to changes of environmental factors and differences in algal communities in relation to land management

were found (Hunt et al., 1979; Metting, 1981; Bérard et al., 2005). However, in these previous studies no cultures have been deposited in culture collections so that a comparison at the molecular genetic level and re-evaluation of the identification of algal taxa is not possible. Additionally comparisons of studies with traditional culturing approaches, based on morphological criteria, are difficult because of different identification strategies of authors and taxonomic uncertainties of floristic lists (Lukešová and Hoffmann, 1996; Neustupa and Škaloud, 2010).

As compared to soil algae, much less is known about algae on tree bark, which are often observed in Europe (Gärtner, 1994; López-Bautista et al., 2007; Freystein et al., 2008; Lüttge and Büdel, 2010; Hodač et al., 2012). Two types of tree bark covers can be noticed; while orchard tree barks were often reddish due to Trentepohliales, the bark of forest trees had often a green cover (Lüttge and Büdel, 2010). The most abundant algae on tree bark may be the cell package forming green alga *Apatococcus*, a member of Trebouxiophyceae (Gärtner and Ingolić, 1989; Gärtner, 1994; López-Bautista et al., 2007). The green biofilms comprise also other common trebouxiophyte genera, e.g. *Trebouxia*, *Coccomyxa*, *Chloroidium* and *Stichococcus* (Freystein et al., 2008; Lüttge and Büdel, 2010). They are also described as food source of microarthropods (Erdmann et al., 2007). Besides morphological studies of corticolous microalgae from tropical habitats (Neustupa and Škaloud, 2008, 2010; Kharkongor and Ramanujam, 2014), also temperate forests were investigated (Mikhailyuk, 1999; Neustupa and Štifterová, 2013). Most green algae found in soil and on tree bark are rather small and inconspicuous so that their identification remains uncertain given the high genetic diversity, e.g. for *Chlorella*-like algae (Rindi et al., 2010; Darienko et al., 2010).

Combined approaches of sequencing of isolates established from communities are still increasing (Lewis and Lewis, 2005; De Wever et al., 2009; Flechtner et al., 2013; Lin et al., 2013; Kulichová et al., 2014; Hodač et al., 2015). Culture-independent approaches with focus on eukaryotic green algal diversity, however, are still lacking. To our knowledge, only one contribution dealing explicitly with diversity of soil algae in a temperate region at the molecular level by cloning and sequencing are available (Bérard et al., 2005). No such studies have been performed for algae on tree barks and no comparison of tree bark algal communities to those in soil. The rRNA gene sequence comparisons provide a clear distinction of taxa independent of plesiomorphic variation and unambiguous re-identification of the genetically same species. The rRNA gene sequence analysis of environmental DNA allows the estimation of genetic diversity of algal communities.

SSU rRNA gene is a commonly used marker molecule; it serves as the gold standard for prokaryotic diversity, but also for estimates of eukaryotic microbial diversity due to the tremendous extent of sequences available for almost all known groups of organisms and its suitability for phylogenetic analyses.

In this study the green algal communities of soil and tree bark from three different management types in spruce and beech forests were investigated and compared. Sampling sites were nine defined research plots of the German Biodiversity Exploratory Schwäbische Alb (Fischer et al., 2010).

Mainly a culture-independent approach was used, i.e. cloning and sequencing of 18S rRNA genes from soil/tree bark DNA extracts. To achieve a maximum recovery of the green algal diversity in the soil, the same soil samples were also used for establishing cultures and the 18S rRNA gene sequences from both approaches were compared. The aim of the present study was to compare the green algal diversities from both habitats, soil and tree bark of the same research plots. This was to test the hypothesis that the diversities of both habitats may largely overlap with the tree bark species richness being lower compared to that of the soil because the latter habitat may be more harsh. In addition, the studied research plots represented three different types of forest management and provided different substrate trees. This allowed to test in as much different intensities of forest management (age class forest *versus* unmanaged forest) and different types of tree bark (beech *versus* spruce) influence the composition of green algal assemblages in soil and on tree bark.

Material and Methods

Study site and sampling

The soil and tree bark samples were collected from nine forest plots of the German Biodiversity Exploratory Schwäbische Alb (48°44'N, 9°39'E) in the low mountain ranges of South-western Germany in the state of Baden-Württemberg. The study sites were investigated within the large-scale and long-term project 'Biodiversity Exploratories' (www.biodiversity-exploratories.de; Fischer et al., 2010).

The studied nine plots represent three different forest and management types with three replicates of each type (Table 1). They included three spruce age class forests (plots AEW1, AEW2, AEW3), three beech age class forests (plots AEW4, AEW5, AEW6) and three unmanaged beech forests (plots AEW7, AEW8, AEW9). Both the spruce and beech

age class forests represent managed sites. The investigated plots comprise Cambisol or Leptosol soil types (Table 1). The soil pH values varied in the spruce plots between 3.3 and 5.04, in the beech plots between 4.5 and 6.4 (for detailed measurements and further physical and geochemical characteristics see Nacke et al., 2011). The soil samples were taken from a given area of 20 m x 20 m from the A-horizons in April and May 2008 with a motor-driven soil column cylinder as described in Will et al., 2010. Samples of tree barks covered by a green biofilm were collected from spruce (*Picea abies* (L.) H. Karst) and beech (*Fagus sylvatica* L.; Table 1), at the same plots in July 2008. Biofilm samples were scraped off from tree bark at a height of about 1.5 m with a sterile scalpel and stored in 2 ml reaction tubes.

Table 1. Sampling sites and numbers of the retrieved green algal OTUs from soil and tree bark of temperate forests.

Sample	Coordinates	Management type	Substrate	Tree/Soil type	Total no. of sequences (algal seq.)	No. of algal OTUs	Coverage* (%)
AEW1	48°28'N, 9°20'E	Age class forest	tree bark	Spruce	7 (5)	5	0
			soil	Cambisol	45 (4)	3	50
AEW2	48°22'N, 9°21'E	Age class forest	tree bark	Spruce	18 (15)	6	80
			soil	Leptosol	102 (61)	16	93.4
AEW3	48°24'N, 9°21'E	Age class forest	tree bark	Spruce	21 (18)	8	72.2
			soil	Cambisol	37 (33)	7	90.9
AEW4	48°23'N, 9°14'E	Age class forest	tree bark	Beech	NA	NA	NA
			soil	Cambisol	51 (38)	10	94.7
AEW5	48°25'N, 9°24'E	Age class forest	tree bark	Beech	31 (29)	4	89.7
			soil	Cambisol	38 (36)	5	97.2
AEW6	48°23'N, 9°26'E	Age class forest	tree bark	Beech	27 (21)	9	81.0
			soil	Cambisol	38 (30)	13	73.3
AEW7	48°23'N, 9°15'E	Unmanaged forest	tree bark	Beech	125 (111)	11	98.2
			soil	Leptosol	39 (38)	4	100
AEW8	48°22'N, 9°22'E	Unmanaged forest	tree bark	Beech	35 (33)	7	87.9
			soil	Cambisol	56 (56)	7	94.6
AEW9	48°22'N, 9°24'E	Unmanaged forest	tree bark	Beech	21 (18)	7	88.9
			soil	Leptosol	34 (29)	3	100

*after GOOD: [1-(n/N)] x 100
n = number of OTUs represented by just one clone, N = total number of clones
NA= data not available

Isolation and microscopy

In order to obtain cultures of green algae one gram of fresh soil material was spread onto plates with agarized (1.5 %) 3NBBM+V culture medium (Starr and Zeikus, 1987) and kept at 18 °C under a light:dark regime of 14:10 hrs at a light intensity of about 25 µE from white fluorescent bulbs. After three to six weeks algal colonies appeared and selected colonies were repeatedly transferred onto new plates until the cultures were unialgal by microscopy. The isolated algae were kept as stock cultures on agar slants (1.5 %) with the same culture medium and under the same growth conditions. Representative isolates were accessioned by the Culture Collection of Algae (SAG; Friedl and Lorenz, 2012) under the strain numbers SAG 2476 - SAG 2483 after phylogenetic analyses. Microscopic observations were accomplished with an Olympus BX60 microscope (Tokyo, Japan) with Nomarski DIC optics with a ColorView III camera (Soft Imaging System, Münster, Germany) attached and micrographs were processed using the Cell^D image software (Soft Imaging System, Münster, Germany).

DNA extraction, PCR amplification, cloning and sequencing

The MoBio PowerSoil DNA isolation Kit (MoBio Laboratories Inc. Carlsbad, CA, USA) was used to extract the genomic DNA from soil samples, i.e. about 10 g of soil from each sample (Will et al., 2010). The Invisorb® Spin Plant Mini Kit (Stratec Molecular, Berlin, Germany) was used for DNA extraction of tree bark biofilm samples and cultures after the cells were disrupted by beating with glass beads (Hallmann et al., 2013a). Extraction results were evaluated after electrophoresis on a 1 % (w/v) agarose gel. Isolated DNA was stored at -20 °C until further processing. For soil and tree bark samples PCR amplification of 18S rRNA genes were conducted in two rounds, first amplified using the eukaryotic specific primers 20F (Hallmann et al., 2013a) and 18L (Hamby et al., 1988) followed by a second amplification with the primers 20F and CH1750R (Hallmann et al., 2013a) which preferentially amplify Chlorophyta rRNA genes. PCR conditions were as described previously (Hallmann et al., 2013a). For algal isolates PCR was conducted directly with the primer pair 20F and CH1750R, using 35 PCR cycles. Aliquots of 2 µl of the PCR products were analysed by electrophoresis on a 1 % (w/v) agarose gel. After purification using the Invisorb® Spin PCRapid Kit (Stratec Molecular, Berlin, Germany) the PCR products were cloned using the TOPO® TA cloning kit (Life Technologies, Carlsbad, CA, USA) with TOP 10 chemically competent one Shot® *Escherichia coli* cells (Life Technologies, Carlsbad, CA, USA). Positive white clones (blue/white screening), grown

on LB agar plates containing 0.1 % ampicillin and 0.2 % X-Gal at 37 °C overnight, were checked for the presence of an insert by vector-specific primers M13F/M13R (Life Technologies, Carlsbad, CA, USA). Positive clones were cultivated overnight in 2 ml reaction tubes with 1 ml LB medium containing 100 µg ampicillin. The plasmid purification kit (Stratec Molecular, Berlin, Germany) was used for plasmid DNA extraction and purification followed by cycle sequencing reaction with the standard sequencing primer 895R (Hallmann et al., 2013a) which yielded partial sequences of about 700 nucleotides (nts) and included the hypervariable regions V2-V4. Purified PCR products from pure cultures were directly used for sequencing. The cycle sequencing reactions were done with the Big Dye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems, Foster city, USA) and the reactions were separated on an ABI Prism 3100 (Applied Biosystems, Foster city, USA) automated sequencer.

Sequence and phylogenetic analyses

The sequences were edited and assembled into contigs using the programme SeqAssem (Hepperle, 2004). Vector sequence was removed and all sequences shorter than 400 bp were excluded from further analyses. The sequences were compared with similar sequences of reference organisms by performing BLASTn searches at NCBI (Altschul et al., 1990, http://www.ncbi.nlm.nih.gov/). Next relative sequences were imported into the ARB program (Ludwig et al., 2004; www.arb-home.de), additional to internal sequence references. To determine the first phylogenetic affiliation the partial sequences were aligned with the homologous eukaryotic 18S rRNA gene sequences by using the automatic alignment tools of the ARB program package. Non-algal sequences were excluded from further analysis. Potential chimeras were examined by Bellerophon (Huber et al., 2004); in addition, the first and the last 300 bp of putative chimeras were compared with similar rRNA gene sequences in NCBI. Chimeric sequences were excluded from the data set. Operational taxonomic units (OTUs), defined at ≥ 98 % sequence similarity (Hallmann et al., 2013a and references therein), were calculated with MOTHUR version 1.13.0 (Schloss et al., 2009). One up to three representative 18S rRNA gene sequences of almost each OTU obtained from environmental preparations and from isolates was sequenced in full length.

The coverage values of the clone libraries were calculated according to Good's formula (Good, 1953), $C = (1- n/N) \times 100$ (C is the percentage of coverage of the library, n the number of singletons and N the total number of clones). A representative selection of

partial and full sequences obtained in this study was deposited in GenBank under the following accession numbers, KP081318 - KP081408. The alignment for phylogenetic analysis was performed using MAFFT version 6 (Katoh and Toh, 2008), alignment was checked for misaligned positions by eye, using BioEdit alignment editor (Hall, 1999). The alignment comprised 194 sequences from the Chlorophyta, i.e. representatives of both classes Trebouxiophyceae and Chlorophyceae, and was 1902 positions (948 variable/698 parsimony informative sites) long. Phylogenies were constructed with the maximum likelihood (ML) method using RAxML (Stamatakis et al., 2008), using the GTR+Γ+I model with 100 replicates. In addition, Bayesian methods were employed with MrBayes 3.2.1 (Huelsenbeck and Ronquist, 2001). Four Markov chains and two million generations sampling every 100 generations were applied, using the GTR+Γ+I model. For calculating pairwise sequence similarities (p-distances) between clone/isolate and reference sequences the program MEGA6 (Tamura et al., 2013) was used.

Statistical analyses

The overall similarities of clone libraries from soils (labelled AEW-B) and those from tree barks (labelled AEW-R) were compared based on the presence/absence matrix of the OTU occurrence. In order to identify clusters of OTUs occurring at similar sampling plots, Principal components analysis (PCA) was conducted with the same dataset. A data subset representing clone libraries only from tree barks (AEW-R) was further analysed for similarities between spruce and beech samples with PCA. All statistical analyses were computed with the program PAST 2.17c (Hammer et al., 2001).

Results

From nine research forest sites of the Exploratory Schwäbische Alb a total of 725 partial 18S rRNA gene sequences were retrieved. Non-algal sequences were excluded from further analysis. Altogether there were 575 sequences representing various green algae from a total of 17 clone libraries obtained from soils (nine) and tree barks (eight) (Table 1). The majority of the sequences (325) represented green algae from soil; 250 were obtained from tree bark samples. The coverage values for each clone library reached an average about 81.9 % (Table 1); combined across all samples the coverage was almost saturated with 96.6 % for soil and with 98.4 % for tree bark. The clone sequences (from soil and tree bark) were distributed on 52 OTUs. In addition, 20 morphologically different isolates were

obtained from soil and six from tree bark samples, sequence analyses of them revealed a total of 17 different OTUs.

Soil algal diversity

The clone libraries of the nine soil samples consisted of 42 OTUs in total. According to the phylogenetic analyses, these OTUs belonged either to the green algal classes Trebouxiophyceae or to Chlorophyceae (Fig. 1 and Fig. 2). Overall 90 % of the clones represented Trebouxiophyceae comprising 30 OTUs, while 10 % belong to Chlorophyceae, comprising 12 OTUs (Table 2). The soil algal richness of the sampling sites varied from three (AEW1 and AEW9) to 16 OTUs (AEW2). The number of OTUs varied between sampling sites of different forest management types. Samples of the age class forests exhibited higher numbers of OTUs, with 22 (spruce; AEW1-3) and 24 OTUs (beech; AEW4-6) than the unmanaged beech forests AEW7-9 which had just about half the number (11) of OTUs (Fig. 3, Table 2). Similarly, for the managed forest plot groups seven and eight OTUs just for the Chlorophyceae were recovered, whereas for the unmanaged plot group just a single chlorophycean OTU was recovered in the soil samples.

The most frequently recovered soil OTU was *Elliptochloris* OTU 10, found in five out of the nine samples, representing 4.3 % of the total number of soil clones. Also discovered in high abundances were *Desmococcus*, *Prototheca*-relative OTU 26 and OTU 29 in four samples and *Apatococcus* OTU 03, *Elliptochloris* OTU 13 and *Prototheca*-relative OTU 28, which occurred in three samples. Three of the most abundant OTUs, the *Prototheca*-relatives (OTUs 26, 28 and 29), represented more than 47 % of all clones retrieved from soil (Table 2). Further seven OTUs (16.7 %) occurred in two sampling sites, whereas 28 OTUs (66.7 %) existed in one sample.

Fig. 1. Phylogenetic tree of Trebouxiophyceae and Chlorophyceae based on 18S rRNA gene sequences with two members of Nephroselmidophyceae as outgroup taxa using maximum likelihood method (ML). The portion of the tree representing the **Trebouxiophyceae** is shown. Thick lines indicate internal nodes that received significant statistical support in both, maximum likelihood and Bayesian analyses (i.e. >75 % bootstrap value with ML and >0.95 Bayesian posterior probabilities). Highlighted sequences (blue) represent full 18S rRNA gene sequences obtained in this study. Sequences in bold indicate representative sequences for a certain of OTUs. An asterisk indicates an OTU represented by the next available reference sequence (see Table S1). Filled circles: OTU from tree bark, empty circles: OTU from soil samples.


CHLOROPHYCEAE
TREBOUXIOPHYCEAE
KF673392 Coccomyxa viridis SAG 216-14 OTU 06
KP081328 Coccomyxa clone AEW7R_K95 OTU 07
AM167525 Coccomyxa glaronensis CCALA 306
KP081391 Coccomyxa isolate SAG 2483 OTU 07
KP081331 Coccomyxa clone AEW2B_K42 OTU 08
KP081332 Coccomyxa clone AEW7R_K132 OTU 08
HE586505 Pseudococcomyxa simplex CAUP H 103
KM020051 Pseudococcomyxa sp. SAG 2253 *OTU 09
AY762603 Coccomyxa pringsheimii SAG 69.80
KP081392 Coccomyxa isolate LH08AW1017 OTU 53
HE617184 Uncultured Trebouxiophyceae isolate RL75K2
HE586514 Coccomyxa sp. KN-2011-T2
KP081337 Elliptochloris clone AEW7R_K114 OTU 10
KP081335 Elliptochloris clone AEW4B_K321 OTU 10
FR865675 Elliptochloris subsphaerica CCAP 211/56
FJ648515 Elliptochloris sp. SAG 2117 OTU 12
KM020164 Hemichloris antarctica SAG 62.90 OTU 11
DQ530055 Elliptochloris bilobata SAG 245.80
KP081342 Elliptochloris clone AEW7B_K389 OTU 13
KP081341 Elliptochloris clone AEW8R_K241 OTU 13
AJ581912 Botryococcus braunii isolate Titicaca
AY762605 Choricystis minor SAG 17.98
KP081344 Chloroidium clone AEW2B_K61 OTU 14
KP081343 Chloroidium clone AEW5R_K23 OTU 14
FM946011 Chloroidium engadinensis SAG 812-1
FM946012 Chloroidium ellipsoideum SAG 3.95
KP081400 Chloroidium isolate LH08AW8042 OTU 14
FM946000 Chloroidium saccharophilum SAG 211-9a *OTU 15
X73998 Heterochlorella luteoviridis SAG 211-2a
X73991 Watanabea reniformis SAG 211-9b
HQ902935 'Myrmecia' sp. CCAP 221/8
KP081398 Lobosphaera isolate LH08AW3064 OTU 20
EU878372 'Parietochloris' cohaerens UTEX 1707
AY762602 Lobosphaera incisa SAG 2007
Z47209 'Myrmecia' bisecta IB T74
KP081351 Lobosphaera clone AEW2B_K6 OTU 20
EU123942 Trebouxia aggregata SAG 219-1d OTU 21
Z68700 Trebouxia jamesii UBT-86.132E2 *OTU 22
Z21551 Trebouxia impressa UTEX 892 OTU 24
KP081355 Trebouxia clone AEW6B_K207 OTU 23
KP081356 Trebouxia clone AEW7R_K112 OTU 23
Z28971 Myrmecia biatorellae UTEX 907
AB080310 Asterochloris erici IAM C-593 OTU 25
JX169826 Apatococcus lobatus SAG 2359 *OTU 04
JX169825 Apatococcus lobatus SAG 2037 OTU 05
KP081318 Apatococcus clone AEW6B_K237 OTU 01
JX169829 Apatococcus lobatus SAG 2151 OTU 02, *OTU 03
KP081319 Apatococcus clone AEW7R_K37 OTU 01
GU017659 Dictyochloropsis irregularis SAG 2036 *OTU 17, *OTU 19
GU017665 Dictyochloropsis splendida UTEX 2599
KP081349 Dictyochloropsis clone AEW1R_K245 OTU 18
M62999 Coleochlamys perforata
Z28974 Microthamnion kuetzingianum UTEX 1914
FJ946881 Trebouxiophyceae sp. SC2-2
HE984579 Leptochlorella corticola I2e
FJ790649 Uncultured Trebouxiophyceae clone Qe17 *OTU 38
KP081399 Unidentified isolate SAG 2477 OTU 58
EU878373 Parietochloris alveolaris UTEX 836
JQ988940 Uncultured Xylochloris clone HEG9B_K2617
JQ988942 Xylochloris isolate SAG 2382 OTU 39
JQ988938 Xylochloris clone AEW2R_K255 OTU 39
EU105209 Xylochloris irregularis CAUP H 7801
GU017662 Dictyochloropsis splendida CAUP H 8601
JQ988930 Dictyochloropsis isolate LH08AW3050 OTU 16
KP081346 Dictyochloropsis clone AEW1B_K153 OTU 16
KP081347 Dictyochloropsis clone AEW7R_K99 OTU 16
GU017664 Dictyochloropsis sp. SAG 2098
EF200531 Prasiola crispa Pcri2
KP081369 Stichococcus clone AEW4B_K337 OTU 33
AJ311637 Stichococcus bacillaris SAG 379-1b OTU 34
KP081368 Desmococcus clone AEW7B_K371 OTU 32
KP081367 Desmococcus clone AEW5R_K28 OTU 32
KM020049 Desmococcus olivaceus SAG 1.92
KP081397 Stichococcus isolate LH08AW8025 OTU 57
AY762604 Trebouxiophyte sp. UR47/4 (SAG 2059)
DQ275460 Stichococcus deasonii UTEX 1706
KP081395 Stichococcus isolate SAG 2482 OTU 56
KF144238 Stichococcus sp. D4-2A
KP081394 Stichococcus isolate SAG 2481 OTU 55
AB055866 Stichococcus bacillaris K4-4
KP081407 Stichococcus isolate KP09AW1004 OTU 55
KF673370 Diplosphaera mucosa SAG 48.86
KP081374 Diplosphaera clone AEW9B_K461 OTU 36
DQ275461 Stichococcus jenerensis D 4
JX185690 Pseudostichococcus monallantoides SAG 380-1
KP081373 Pseudochlorella clone AEW7R_K128 OTU 35
AM422985 Pseudochlorella pyrenoidosa SAG 18.95
KM020138 Pseudochlorella pringsheimii SAG 211-1a
JX169834 Coenochloris signiensis CCAP 176/3
X74000 'Chlorella' mirabilis Andreyeva 748-I *OTU 40
AB017435 Coenocystis inconstans
KP081393 Neocystis isolate SAG 2480 OTU 37
KM020044 Neocystis brevis SAG 850-1
JQ920367 Neocystis mucosa SAG 40.88
KF144212 Neocystis cf. mucosa SAG 2405
KP081360 Prototheca-rel. clone AEW5B_K253 OTU 26
KP081365 Prototheca-rel. clone AEW7B_K364 OTU 30
KP081362 Prototheca-rel. clone AEW3B_K182 OTU 27
KP081364 Prototheca-rel. clone AEW5B_K252 OTU 29
X63519 Prototheca zopfii SAG 263-1a
AB470468 Prototheca cutis asahi57
KP081366 Prototheca-rel. clone AEW3B_K185 OTU 31
X74003 Prototheca wickerhamii SAG 263-11
X56101 Auxenochlorella protothecoides SAG 211-7a
KP081390 Auxenochlorella isolate SAG 2478 OTU 54
KJ616755 Chlorella sorokiniana LB65
FM205832 Chlorella vulgaris SAG211-11b OTU 59
AB012845 Muriella terrestris ASIB V38
AB080300 Nannochloris bacillaris
X68484 Scheffelia dubia
X70802 Tetraselmis striata Ply 443
AF125167 Picocystis salinarum SSFB
X75565 Nephroselmis pyriformis CCMP 717
X74754 Nephroselmis olivacea SAG 40.89
0.05
Choricystis/ Botryococcus-clade
Watanabea-clade
Lobosphaera-clade
Trebouxia-clade
Prasiola-clade
Chlorellales
Chlorodendrophyceae
Nephroselmidophyceae

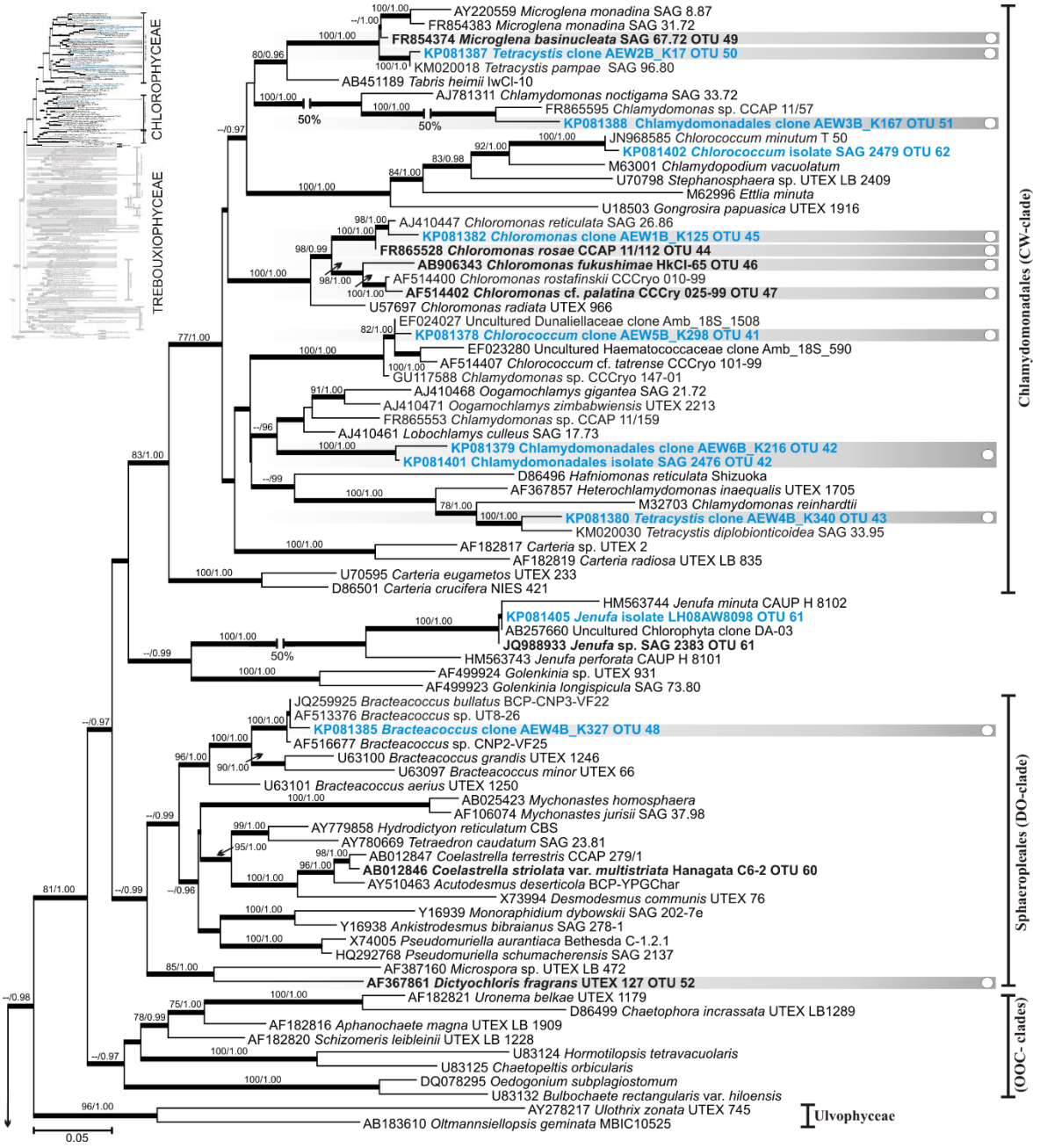

Fig. 2. Phylogenetic tree of Trebouxiophyceae and Chlorophyceae based on 18S rRNA gene sequences with two members of Nephroselmidophyceae as outgroup taxa using maximum likelihood method (ML). The portion of the tree representing the **Chlorophyceae** is shown. Thick lines indicate internal nodes that received significant statistical support in both, maximum likelihood and Bayesian analyses (i.e. >75 % bootstrap value with ML and >0.95 Bayesian posterior probabilities). Highlighted sequences (blue) represent full 18S rRNA gene sequences obtained in this study. Sequences in bold indicate representative sequences for a certain of OTUs. Filled circles: OTU from tree bark, empty circles: OTU from soil samples.

Table 2. Distribution of 52 green algal OTUs in the 18S rRNA gene clone libraries from the soil and tree bark of temperate forests. The OTUs belong to the green algal classes of Trebouxiophyceae and Chlorophyceae. An asterisk indicates an isolate is available for this OTU.

		Soil									Tree bark								
OTU name	OTU no.	AEW1	AEW2	AEW3	AEW4	AEW5	AEW6	AEW7	AEW8	AEW9	AEW1	AEW2	AEW3	AEW5	AEW6	AEW7	AEW8	AEW9	
Apatococcus	OTU 01	-	-	-	-	-	1	-	-	-	-	-	-	-	-	28	1	-	OTUs from soil and tree bark
Apatococcus	OTU 03	-	4	-	-	-	1	-	1	-	-	1	-	-	1	1	-	-	
Apatococcus	OTU 05	2	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	
Chloroidium	OTU 14*	-	3	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	
Coccomyxa	OTU 06	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	12	2	
Coccomyxa	OTU 07*	-	6	-	-	-	-	-	-	-	1	4	6	-	2	13	14	4	
Coccomyxa	OTU 08	-	2	-	-	-	1	-	-	-	-	5	1	-	-	22	-	5	
Desmococcus	OTU 32	-	-	-	-	3	2	15	4	-	-	-	-	26	-	-	-	1	
Dictyochloropsis	OTU 16*	1	3	-	-	-	-	-	-	-	1	-	-	-	-	26	1	-	
Elliptochloris	OTU 10	-	5	1	2	-	1	5	-	-	-	-	1	1	1	10	1	-	
Elliptochloris	OTU 13	-	4	1	-	-	-	15	-	-	1	-	-	-	-	2	1	-	
Hemichloris	OTU 11	-	-	2	-	-	-	-	-	-	-	1	-	-	-	-	-	-	
Trebouxia	OTU 22	-	-	-	-	-	1	-	-	-	-	-	-	-	3	-	-	-	
Trebouxia	OTU 23	-	-	-	-	-	2	-	-	-	-	-	-	-	2	1	-	1	
Apatococcus	OTU 04	-	-	-	-	-	-	-	-	-	-	-	-	-	8	3	3	3	OTUs only from tree bark
Asterochloris	OTU 25	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	
Coccomyxa	OTU 09	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	2	
Dictyochloropsis	OTU 17	-	-	-	-	-	-	-	-	-	1	-	2	-	-	-	-	-	
Dictyochloropsis	OTU 18	-	-	-	-	-	-	-	-	-	1	-	-	1	1	-	-	-	
Dictyochloropsis	OTU 19	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	
Pseudochlorella	OTU 35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	
Trebouxia	OTU 21	-	-	-	-	-	-	-	-	-	-	1	-	-	2	-	-	-	
unident. trebouxiophyte	OTU 40	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-	
Xylochloris	OTU 39*	-	-	-	-	-	-	-	-	-	-	3	1	-	-	-	-	-	
Apatococcus	OTU 02	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	OTUs only from soil
Bracteacoccus	OTU 48	-	1	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	
Chlamydomonadales	OTU 42*	-	1	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	
Chlamydomonas	OTU 51	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Chlorococcum	OTU 41	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	
Chloroidium	OTU 15	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Chloromonas	OTU 46	-	6	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	
Chloromonas	OTU 44	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	
Chloromonas	OTU 45	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Chloromonas	OTU 47	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	
Dictyochloris	OTU 52	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	
Diplosphaera	OTU 36	-	-	-	-	-	-	-	-	9	-	-	-	-	-	-	-	-	
Elliptochloris	OTU 12	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	
Lobosphaera	OTU 20*	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Microglena	OTU 49	-	-	-	6	-	-	-	-	-	-	-	-	-	-	-	-	-	
Neocystis	OTU 37*	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	
Prototheca	OTU 31	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Prototheca-relative	OTU 26	-	18	17	-	16	13	-	-	-	-	-	-	-	-	-	-	-	
Prototheca-relative	OTU 27	-	-	6	-	-	3	-	-	-	-	-	-	-	-	-	-	-	
Prototheca-relative	OTU 28	-	-	-	2	-	-	-	2	6	-	-	-	-	-	-	-	-	
Prototheca-relative	OTU 29	-	-	-	6	14	-	-	45	14	-	-	-	-	-	-	-	-	
Prototheca-relative	OTU 30	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	
Stichococcus	OTU 34	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	
Stichococcus	OTU 33	-	-	-	11	-	-	-	-	-	-	-	-	-	-	-	-	-	
Tetracystis	OTU 43	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	
Tetracystis	OTU 50	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Trebouxia	OTU 24	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	
unident. trebouxiophyte	OTU 38	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Total no. of clones		4	61	33	38	36	30	38	56	29	5	15	18	29	21	111	33	18	
Total no. of OTUs		3	16	7	10	5	13	4	7	3	5	6	8	4	9	11	7	7	

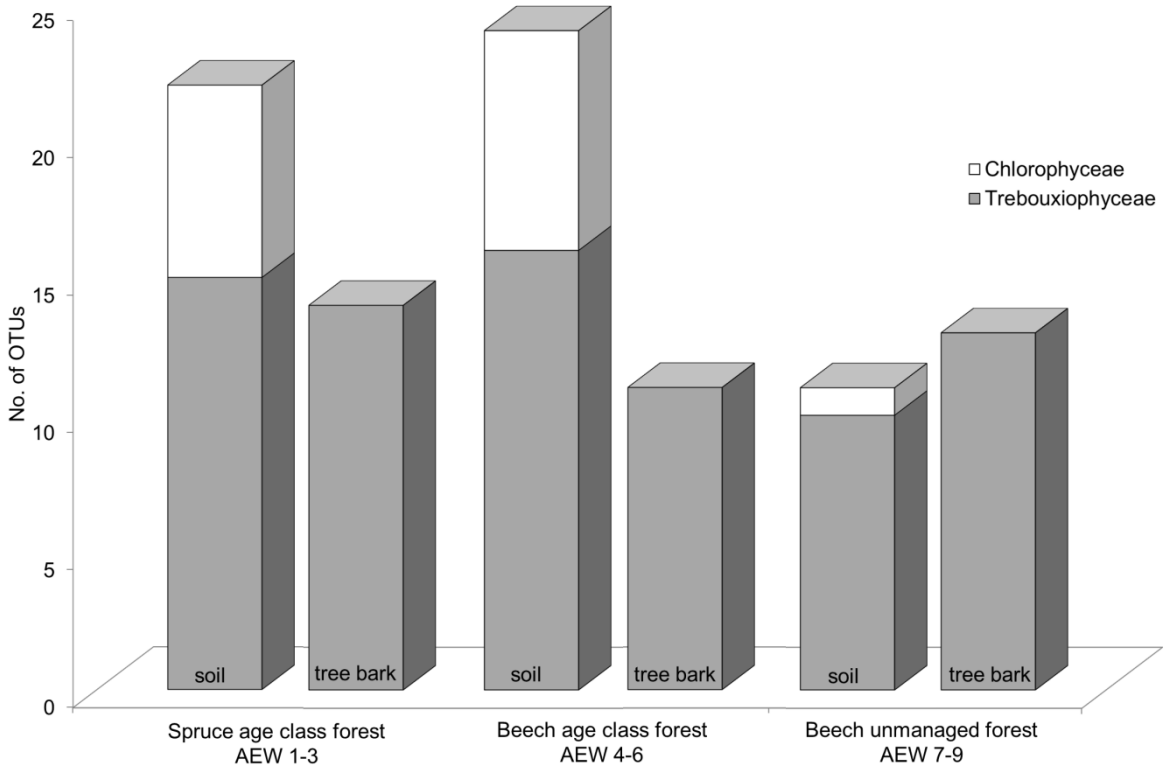

Fig. 3. Distribution of 52 green algal OTUs in the 18S rRNA gene clone libraries from the soil and tree bark at different forest management types.

Culturing increased the number of OTUs recovered from the soil by 20 cultures (16 different OTUs), i.e. to a total of 52 OTUs (Table 3). With the cultures, there were seven additional OTUs from the Trebouxiophyceae and three OTUs for the Chlorophyceae which exclusively were recovered by the cultures, but not by the culture-independent cloning approach. Both approaches overlapped in no more than six OTUs (12 % of all recovered OTUs), whereas 36 OTUs (69 %) were exclusively retrieved by the culture-independent approach (Table 2).

Five trebouxiophycean OTUs (OTUs 07, 14, 16, 20, and 37) were concordantly detected by both approaches and exhibited partially a higher inner phylogenetic diversity. The sequences representing OTU 16 (including that of isolate LH08AW3050) had high similarities of 99.9-100 % with *Dictyochloropsis splendida* (reference sequence JQ988930) and, therefore, were identified as this species. Isolate LH08AW3050 was also characterized by a morphology characteristic for that species, i.e. spherical cells with a rich and finely lobed chloroplast without a visible pyrenoid (Fig. 4a). Similarly, sequences

representing OTU 14 had high sequence similarities of 99.1-99.3 % with the authentic strain of *Chloroidium saccharophilum*, SAG 211-9a. The isolate LH08AW8042 from that OTU had even a sequence 100 % identical with SAG 211-9a and, therefore, was representing that species. Similarly, the isolate representing OTU 07 (strain SAG 2483) had a 100 % identical sequence with reference sequence AM167525 representing *Coccomyxa glaronensis*. The full clone sequence representative for this OTU (AEW7R_K95) had 99 % sequence similarity with the authentic strain of *C. viridis*, SAG 216-14. Therefore, OTU 07 may represent even several species of *Coccomyxa*. Correspondingly, the OTU 20 isolate (LH08AW3064) shared 100 % sequence identity with strain CCAP 221/8 assigned to '*Myrmecia'* sp., whereas the representative full clone sequence (clone AEW2B_K6) was 99.9 % identical with the corresponding sequence of the authentic strain of '*M.' bisecta* SAG 2043. Most likely, OTU 20 represented several species of '*Myrmecia'*. The isolate LH08AW3064 exhibited spherical cells with one band-shaped chloroplast without a pyrenoid (Fig. 4b) which are typical features of *Lobosphaera*-like green algae. The isolate for OTU 37 (strain SAG 2480) shared 99.9 % sequence identity with the authentic strain of *Neocystis brevis* SAG 850-1, whereas the representative partial clone sequence for this OTU shared just 98.9 % sequence similarity with isolate SAG 2480.

Seven trebouxiophycean OTUs (OTU 39, OTUs 53-58) were retrieved exclusively by cultures (Table 3). The isolates affiliated to *Coccomyxa* OTU 53 and *Stichococcus* OTU 55/OTU 56/OTU 57 exhibited high similarities (99.9 % to 100 %) to sequences available in GenBank (Fig. 1, Table S1). The *Stichococcus* isolate SAG 2482, representing OTU 56, exhibited a typical *Stichococcus* morphology with rod-shaped cells with a single band-shaped chloroplast (Fig. 4c). The *Auxenochlorella* isolate SAG 2478 (KP081390) of OTU 54 had a 99.9 % sequence similarity with a lichen photobiont strain (AM260449) assigned to this genus. The phylogenetic analyses showed isolate SAG 2478 being a close relative to the authentic strain of *A. protothecoides* SAG 211-7a (Fig. 1). The isolate SAG 2478 exhibited a rather simple morphology, spherical *Chlorella*-like cells, under culture conditions without visible photosynthetic pigments (Fig. 4d). SAG 2382 was within the same OTU 39 as representative fully sequenced clone AEW2R_K255 and reference sequence EU105209 for *Xylochloris irregularis*, the type of the genus *Xylochloris*. Therefore, OTU 39 represents *Xylochloris*. However, the genetic distances of the former two sequences with that of *X. irregularis* as seen in the phylogenetic analyses (Fig. 1) makes it questionable, whether both sequences belong to *X. irregularis*.

Table 3. Green algal isolates retrieved from soil and tree bark of temperate forests. The 17 OTUs belong to the green algal classes of Trebouxiophyceae and Chlorophyceae. An asterisk indicates clones available for this OTU.

OTU Name	OTU No.	Isolate	Acc. No.	Plot	Substrate
Trebouxiophyceae					
Auxenochlorella	OTU 54	SAG 2478	KP081390	AEW4	soil
Chlorella	OTU 59	KP09AW8003	KP081408	AEW8	tree bark
		KP09AW8002	-	AEW8	tree bark
Chloroidium	OTU 14*	LH08AW8042	KP081400	AEW8	soil
Coccomyxa	OTU 07*	SAG 2483	KP081391	AEW8	soil
	OTU 53	LH08AW1017	KP081392	AEW10	soil
Dictyochloropsis	OTU 16*	LH08AW3050	JQ988930	AEW3	soil
		SAG 2305	GU017658	AEW3	tree bark
Lobosphaera	OTU 20*	LH08AW3064	KP081398	AEW3	soil
Neocystis	OTU 37*	SAG 2480	KP081393	AEW8	soil
Stichococcus	OTU 55	SAG 2481	KP081394	AEW8	soil
		KP09AW1004	KP081407	AEW10	tree bark
		KP09AW1006	-	AEW10	tree bark
		KP09AW1001	-	AEW10	tree bark
	OTU 56	SAG 2482	KP081395	AEW8	soil
		LH08AW8104	KP081396	AEW8	soil
	OTU 57	LH08AW8025	KP081397	AEW8	soil
unident. trebouxiophyte	OTU 58	SAG 2477	KP081399	AEW3	soil
Xylochloris	OTU 39*	SAG 2382	JQ988942	AEW7	soil
Chlorophyceae					
Chlamydomonadales	OTU 42*	SAG 2476	KP081401	AEW1	soil
Chlorococcum	OTU 62	SAG 2479	KP081402	AEW5	soil
		LH08AW5111	KP081403	AEW5	soil
		LH08AW5107	KP081404	AEW5	soil
Coelastrella	OTU 60	LH08AW4118	KP081406	AEW4	soil
Jenufa	OTU 61	LH08AW8098	KP081405	AEW8	soil
		SAG 2383	JQ988933	AEW8	soil

Rather, both our OTU 39 sequence from soils of the AEW plots may represent two additional species of *Xylochloris*. Four more trebouxiophycean OTUs were recovered exclusively by cultured isolates, i.e. OTUs 55, 56, 57, and 58 (Tables 3 and S1). The former three exhibited *Stichococcus*-like morphological features (e.g. OTU 56; Fig. 4c) and were also close relatives with reference strains representing various lineages of *Stichococcus* which appeared as a paraphyletic assemblage within the *Prasiola*-clade of Trebouxiophyceae (Fig. 1). Strain SAG 2477 (OTU 58) represented an independent lineage within the Trebouxiophyceae. It was somehow closer related to the *Leptochlorella*-clade, but there was no statistical support for this relationship (Fig. 1).

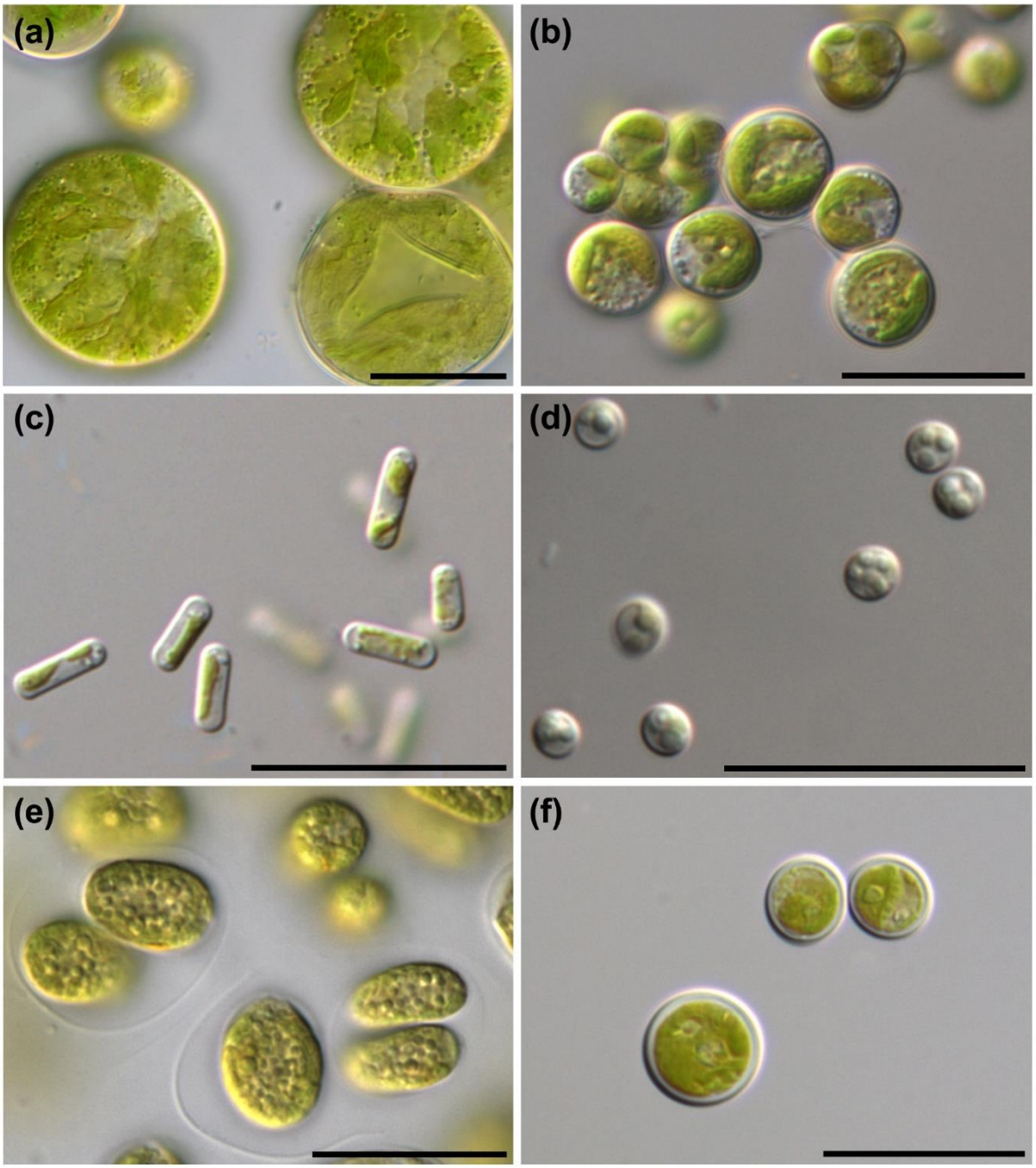

Fig. 4. Photomicrographs of cultured isolates. (a) - (d) Trebouxiophyceae, (e) - (f) Chlorophyceae. (a) *Dictyochloropsis* sp. LH08AW3050 (OTU 16); (b) '*Myrmecia'* sp. isolate LH08AW3064 (OTU 20); (c) *Stichococcus* sp. isolate SAG 2482 (OTU 56); (d) *Auxenochlorella* sp. isolate SAG 2478 (OTU 54); (e) Chlamydomonadales sp./*Oogamochlamydinia* clade isolate SAG 2476 (OTU 42); (f) *Chlorococcum* cf. *minutum* isolate SAG 2479 (OTU 62). Scale bars, 20 μm.

Only one member of the class Chlorophyceae was found by both culturing and cloning. The OTU 42 comprising isolate SAG 2476 and clone AEW6B_K216, was phylogenetically affiliated to Chlamydomonadales sp. /*Oogamochlamydinia* clade and exhibited a distinct linage without known close relatives (Fig. 2). The isolate has characteristic monadoid morphology with elliptic cells with two flagella, one cup-shaped chloroplast (without pyrenoid) and stigma (Fig. 4e). Three more chlorophycean OTUs (OTUs 60, 61, and 62) were detected exclusively with cultures from soils of plots AEW4, AEW 8 and AEW 5 (Tables 3, S1; Fig. 4f). The OTUs 60 and 61 may represent species of *Coelastrella* and *Jenufa*, respectively (Fig. 2; Hodač et al., 2012). OTU 62 has a *Chlorococcum*-like morphology (Fig. 4f), but *Chlorococcum* appears as a polyphyletic assemblage in molecular phylogenies (Fig. 2) and, therefore, the correct assignment of strain SAG 2479 remains unclear at present.

Tree bark algal diversity

From the tree bark samples, collected from two different host trees (*Picea abies* and *Fagus sylvatica*), a total of 24 OTUs were recovered through cloning. All OTUs detected in these samples were affiliated to Trebouxiophyceae, no Chlorophyceae were observed (Table 2, Table S1). The most frequent OTU detected on tree bark was *Coccomyxa* OTU 07, which occurred in seven out of eight samples and represented 17.6 % of the retrieved tree bark clones. Additionally observed in high abundances was *Elliptochloris* OTU 10 in five samples and *Coccomyxa* OTU 08 and *Apatococcus* OTU 04 in four. Twelve OTUs were found twice or more times and eight OTUs were recovered only a single time (Table 2). The highest species/OTU richness of tree bark samples was detected in the plots AEW7 and AEW6 (11 and nine OTUs), both host trees were beech, while lowest species/OTU richness were investigated in AEW1 (spruce) and AEW5 (beech) with five and four OTUs.

No correlation between species/OTU richness on tree barks and forest management types was found (Fig. 3). The number of OTUs retrieved from the different management types were almost the same, from age class forests 14 (spruce) and 11 OTUs (beech) were recovered, from unmanaged beech forests a total of 13 OTUs were retrieved (Fig. 3). Modest differences between OTU richness of spruce (exclusively six OTUs) and beech tree bark (exclusively ten OTUs) were obvious, whereas 33 % (eight OTUs) were common to both host tree species. A separate PCA ordination focusing solely on tree bark samples illustrated dissimilarity in OTU composition of spruce (AEWR1 to AEW3) and beech

(AEWR5 to AEW9) forest samples (Fig. 5). Here, *Dictyochloropsis* OTU 19, *Asterochloris* OTU 25 and *Chlorella mirabilis* relative OTU 40 were detected predominantly on spruce tree barks, whereas, for example, *Trebouxia* OTU 23 or *Apatococcus* OTU 04 occurred more frequently on beech tree barks.

Six isolates were retrieved from tree bark samples (Table 3). The isolate SAG 2305 *Dictyochloropsis splendida* (GU017658), isolated from spruce tree bark, belonged to the same OTU 16, which was found as an isolate and as clones in soil samples. Another three isolates from spruce tree bark were represented by partial sequences and affiliated to *Stichococcus* OTU 55, isolated from soil samples as well. Finally, two isolates belonged to OTU 59, with high similarity (99.8 %) to FM205832 *Chlorella vulgaris* strain SAG 211-11b (isolated from beech tree bark).

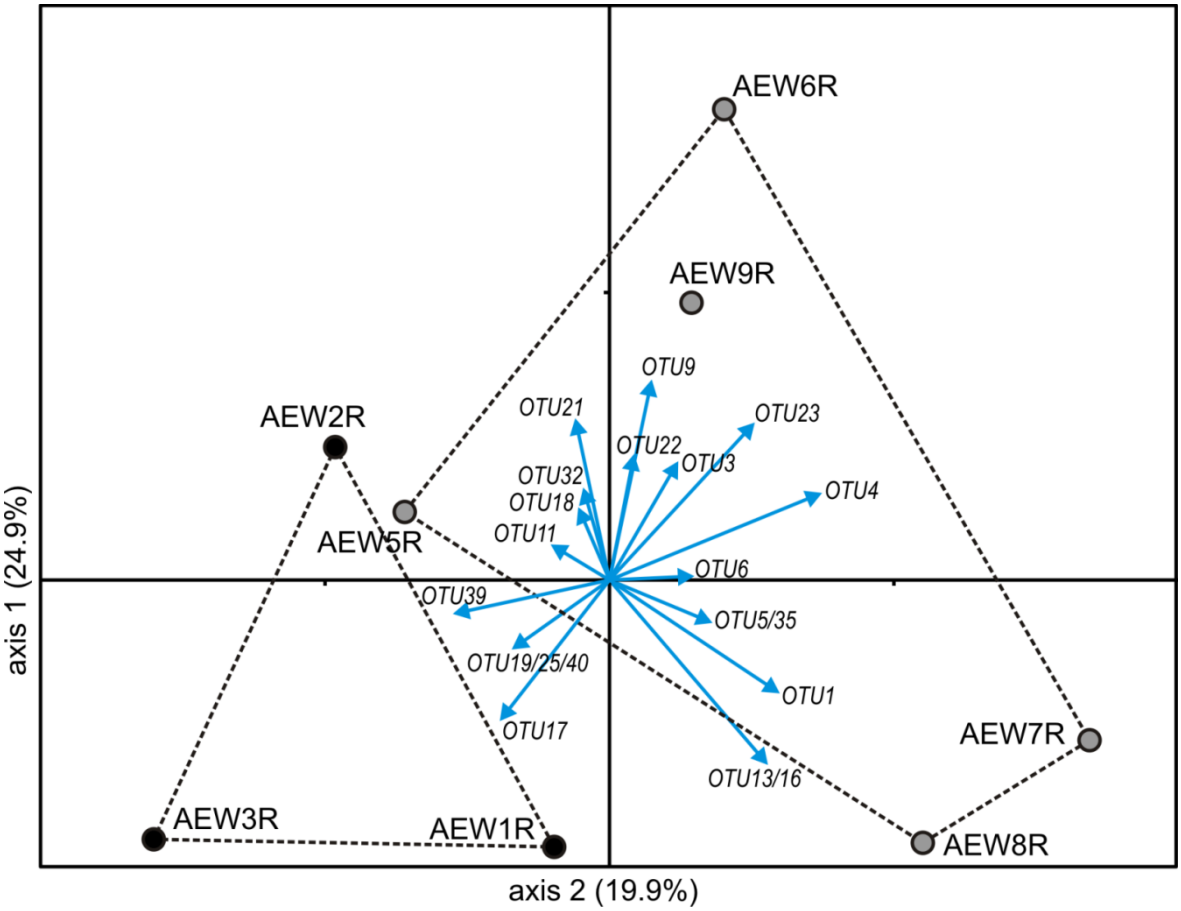

Fig. 5. Principal Components Analysis (PCA). Ordination diagram showing trebouxiophyte OTUs (blue arrows) which are responsible for the main differences between tree-bark samples from spruce (black dots) and beech (grey dots) forests. For the species/genus assignments of the OTUs see Table 2.

Comparison of tree bark algal diversity with that of soil algae

The clone libraries from tree bark samples exhibited lower green algal OTU diversity compared to those from soils. A total of ten OTUs (19 %) were exclusively retrieved from tree bark, 28 OTUs (54 %) were exclusively detected in soil (16 trebouxiophycean OTUs and 12 chlorophycean OTUs), whereas 14 OTUs (27 %) were shared by both habitats (Table 2). The species/OTU richness indicated no major differences between the habitats; the number of OTUs of tree barks samples varied from four to 11 OTUs with an average of 7.1 OTUs and from three to 16 OTUs with an average of 7.6 OTUs for the soil samples (Table 2). In addition to the OTUs affiliated to Chlorophyceae, also some OTUs represented Trebouxiophyceae exhibited habitat preferences. The PCA ordination depicted those trebouxiophycean OTUs which occurred preferentially either in soil or in tree bark samples (Fig. 6). As example, *Coccomyxca* OTU 07, *Coccomyxa* OTU 08 and

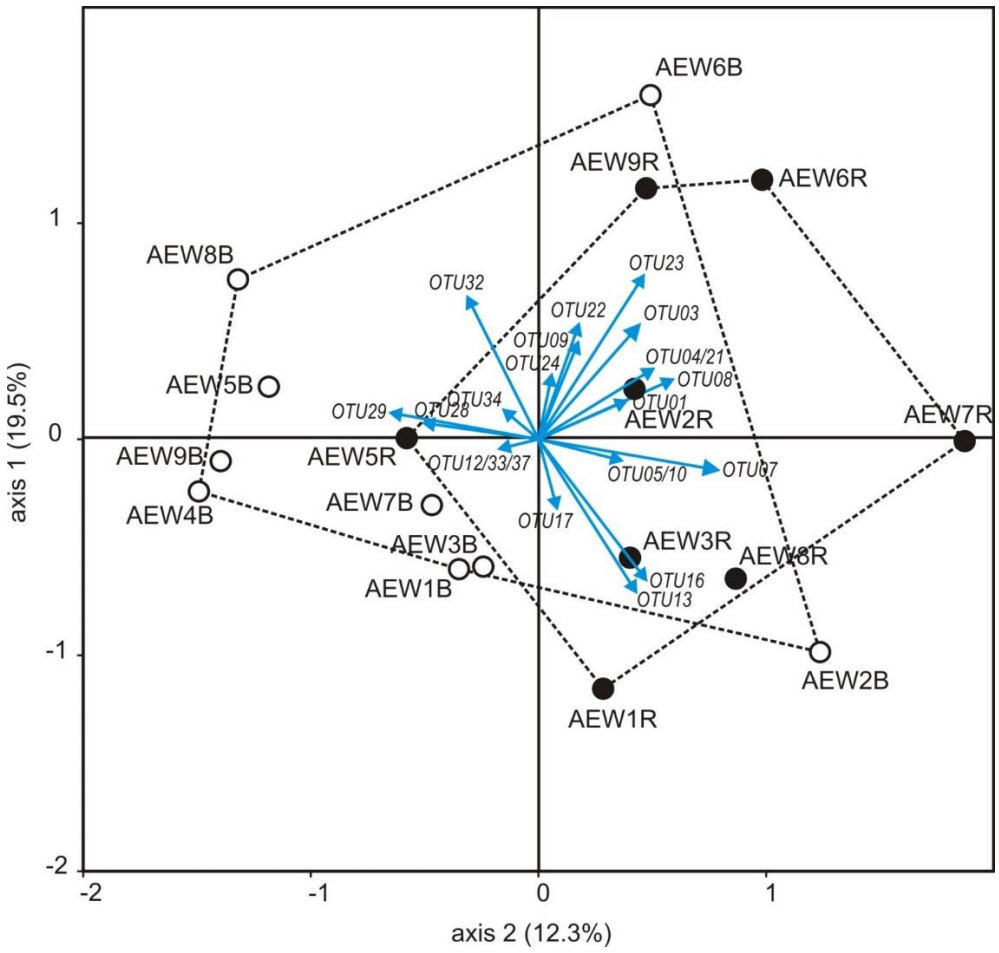

Fig. 6. Principal Components Analysis (PCA). Ordination diagram showing trebouxiophyte OTUs (blue arrows) which correspond to the main differences between soil (”B”, white dots) and tree-bark (”R”, black dots) plots. For the species/genus assignments of the OTUs see Table 2.

Trebouxia OTU 23 represented taxa occurring preferentially on tree barks, whereas the *Desmococcus* OTU 32 occurred more frequently in soils. Clear habitat preferences were shown by *Prototheca*-relative OTUs 26, 27, 28 and 29, which were detected two to four times in high clone numbers from soil, but not a single time from tree bark and *Apatococcus* OTU 04 which was recovered four times from tree bark, but not a single time from soil.

Discussion

In this study the green algal communities from soil and tree bark samples from nine research plots of temperate forests in Germany were investigated through a culture-independent approach and 18S rRNA gene sequence comparisons. The soil samples were additionally analysed by a traditional culturing approach and a few isolates were obtained from tree bark. So far culture-independent approaches including eukaryotic phototrophs from soil were predominantly performed for extreme environments like soils from Antarctic, fumaroles, Himalaya and glacier forefront (Lawley et al., 2004; Costello et al., 2009; Schmidt et al., 2011; Frey et al., 2013). Here we present the first molecular survey on green algae from temperate forests in Central Europe.

Culture-independent and culture-dependent approach

For the culture-independent approach and the sequencing of cultures the PCR primer CH1750R was used that preferentially amplifies 18S rRNA genes from green algae (Hallmann et al., 2013a) in order to enrich the clone libraries, especially of soil samples, with green algal sequences. Clone libraries from soil samples with standard PCR primer combinations which generally amplify eukaryotic 18S rRNA genes (e.g. primer 20F/18L) yielded various sequences from other soil organisms, e.g. fungi, alveolates and nematodes, and these were even dominating over corresponding green algal sequences. In the majority of the established clone libraries green algal 18S rRNA gene sequences were predominant, except for sample AEW1 a large fraction of a *Dileptus*-like ciliate 18S rRNA gene sequence (98 % blast similarity with *Dileptus* sp.; acc. no. AF029764) was retrieved (Table 1). Probably this ciliate was so abundant in AEW1 that it masked the green algae present there. In addition, members of Klebsormidiophyceae and the ulvophyte *Trentepohlia* were not amplified, whilst microscopic observation of soil and tree bark

samples recovered also algae of other groups like *Klebsormidium* sp. (Streptophyta) and Xanthophyceae (Stramenopiles).

For the soil samples we used two approaches, culture-independent rRNA gene cloning/sequencing and sequencing of unialgal cultures developed from the same samples. This allows comparing the algal diversities recovered by both approaches. The cloning approach revealed a higher OTU diversity (42 OTUs) like the culture-based approach (16 OTUs), as expected. Many studies have shown that sequencing of SSU rRNA genes and culture-independent approaches resulted in much higher diversity (e.g. López-García et al., 2001; Moon-van der Staay et al., 2001; Scheckenbach et al., 2010; Lara et al., 2011). Only six OTUs (11.5 % of all detected) were shared by both approaches. Interestingly, these OTUs occurred only in small clone numbers (1-6 clones per OTU). Remarkably no isolate was obtained from the most abundant OTUs retrieved by the cloning approach, i.e. seven OTUs occurred in three up to five samples with relatively high clone numbers from 9 to 69. The different results of samples analyzed by a combination of culture-independent and dependent methods, were often observed (Norris and Castenholz, 2005; Vaz-Moreira et al., 2011; Bazin et al., 2014). Culture-based approaches often favor ubiquitous algae and suppress specialists, also very rare species and non-cultivable algae were not captured (Hoffmann, 1989; Neustupa and Škaloud, 2010).

Diversity of OTUs

The majority of OTUs belonged to the green algal class Trebouxiophyceae and was distributed over five well known distinct clades and various single lineages, representing predominantly aerophytic or soil algae (Friedl and Rybalka, 2012).

The most abundant OTU, *Elliptochloris* OTU 10, found in ten samples (Table 2), exhibited no preference towards tree bark or soil and shared 99.3 % to 99.8 % pairwise sequence similarities, calculated from p-distances (Table S1), to *Elliptochloris subsphaerica* FR865675 isolated from Antarctic soils. *E. subsphaerica* were also observed on tree bark (Ettl and Gärtner, 1995; Freystein et al., 2008). The other *Elliptochloris* OTU, OTU 13, was also without preference towards tree bark or soil because it was recovered from three samples of each habitat (Table 2). The representative clone sequences exhibited only 97.2 % to reference sequence *E. subsphaerica* FR865675. In the phylogenetic tree (Fig. 1) reference strains for all so far known species of *Elliptochloris* were included, but the OTU 13 did not match any of them. Therefore, *Elliptochloris* OTU 13 may represent a still undescribed new species of *Elliptochloris*. As well *Apatococcus* OTU 03 indicated no

preference towards tree bark or soil (found in three samples of each habitat). However *Apatococcus* OTU 04 exhibited a clear preference towards tree bark and was not retrieved from soil. The OTUs affiliated to *Apatococcus* exhibited high similarities (99.1 % to 99.9 %) to reference strains SAG 2037, SAG 2359 and SAG 2151 (JX169825, JX169826, JX169829), isolated from green biofilms collected from tree bark and plastic surfaces. *Apatococcus* was also observed in soil (Ettl and Gärtner, 1995), but this cannot be clearly validated since no *Apatococcus* strains isolated from soil were available (two strains mentioned in Gärtner and Ingolić, 1989 were misidentified, see e.g. sequence KM020045). Characteristic tree bark algae were *Coccomyxa* sp., *Dictyochloropsis*-related and *Trebouxia* sp. because they were found at more plots on tree bark than in the soil. The representative clone sequence for OTU 07, indicated 99 % similarity to the authentic strain *Coccomyxa viridis* SAG 216-14 (KF673392), whereas the representative sequence for OTU 08 AEW7R_K132 showed just 97.5 % and 97.4 % similarities to *Pseudococcomyxa* (HE586505, KM020051). The OTU *Coccomyxa*-related (OTU 08) was nested within a clade representing strains of *Coccomyxa* and *Pseudococcomyxa;* it may represent a species of one of these genera that has not been sequenced so far or a new undescribed species (Fig. 1). OTU 16 was shared by all approaches and was retrieved from tree bark and soil; it is assigned to *Dictyochloropsis splendida.* Interestingly, the genus *Dictyochloropsis* appeared as a paraphyletic assemblage, i.e. *D. splendida* was not closer related to other species of the same genus (Fig. 1). For OTUs, observed in the clone libraries only in one sample, it remained unclear whether they had a habitat preference or not. The OTU affiliated to *Desmococcus olivaceus* (99.8 % and 100 % similarity) could be denoted as a characteristic soil alga because it was retrieved in more samples from soil than from tree bark. *Desmococcus* is well known from tree bark, stone and soil (Gärtner and Ingolić, 2003). The trebouxiophycean OTU 38 as represented by clone AEW2B_K3 (partial sequence KP081376) was a close relative to *Leptochlorella corticola* (Fig. 1). The reference strain for *L. corticola* (sequence HE984579) was, however, not within the same OTU 38. Therefore, identification of the OTU still remains unclear; it may represent another yet undescribed species of *Leptochlorella*. Interestingly, the representative clone was next related to a sequence of a green algal clone recovered from the hypolithic habitat in Tibet (sequence FJ790649; Wong et al., 2010) which, however, shared only 97.9 % similarity with our clone AEW2B_K3 (Table S1). OTU 20 was represented by one isolate (LH08AW3064) and a full length sequence of clone AEW2B_K6 (Fig. 1), next related to '*Myrmecia*' sp. CCAP 221/8 (HQ902935) and '*Myrmecia*' *bisecta* IB T74 (Z47209). This

OTU included as well the reference strains '*Parietochloris*' cohaerens UTEX 1707 (EU878372) and *Lobosphaera incisa* SAG 2007 (AY762602), the only authentic strain of a genus within this clade. Since of the distant position of *L. incisa* to the 'true' *Myrmecia* spp., see *Myrmecia biatorella*, closely related to *Trebouxia* spp. (Friedl, 1995; Karsten et al., 2005), our sequences could be probably assigned to *Lobosphaera* (Fig. 1).

No closer affiliation to any known green algal genera could be found for the five trebouxiophycean OTUs *Prototheca*-relative (OTU 26 to 31). Blast searches revealed just 80 % to 84 % similarities to representatives of Chlorellales or 93 % to AB470468 *Prototheca cutis*. The phylogenetic analysis assigned these sequences within in Chlorellales clade, they had a sister group-relationship with *P. zopfii*, *P. cutis* and *Auxenochlorella protothecoides*, but were separated by long internal branches from the latter three (Fig. 1). Whereas *Prototheca*-relatives OTU 26 to 30 were closer related to *P. zopfii* X63519, OTU 31 was somehow closer related to *P. cutis* AB470468. Interestingly the most abundant OTUs in soil, *Prototheca*-relative OTU 26 to 31, were neither retrieved from tree bark nor could an isolate be established. These OTUs were next related to the heterotrophic genus *Prototheca* (including *A. protothecoides*) which is characterized by an accelerated mutation rate of its 18S rRNA gene, which is indicated by unusually long branches. This is especially obvious for *P. zopfii* (Huss et al., 1999). *Prototheca* species are non-photosynthetic, known as pathogens but also described in soil (Roesler and Hensel, 2003; Ueno et al., 2003). It is likely to assume that the *Prototheca*-relative OTUs were important soil algae and were up to now retrieved by culturing.

The OTUs assigned to Chlorophyceae were distributed on Chlamydomonadales and Sphaeropleales. Most likely, the chlamydomonadalean OTUs (obtained exclusively from soil samples) represent flagellated taxa like *Chlamydomonas* or *Chloromonas*.

Green algal communities in soils and on tree barks

Altogether the soil algal communities exhibited a higher diversity than the communities on tree bark. This may be explained by that soil represents a more heterogeneous habitat, with trickles and small water pits. However in the tree bark samples no chlorophycean OTUs were detected. A similar finding has been reported for tree bark samples in Slovenia and Italy (Kulichová et al., 2014) as well as for sun-exposed stone substrates (Hallmann et al., 2013a, 2013b). Previous studies observed a few findings of Chlorophyceae on tree bark, but mainly resting stages like palmelloid phases of *Chlamydomonas* (Gärtner, 1994). Light, humidity, temperature, availability of nutrients, and pH seem to be the most important

environmental factors that influence soil algal communities (Metting, 1981; Hoffmann, 1989). Based on the factors humidity and light it is conceivable to obtain exclusively in soil Chlorophyceae with motile members like *Chlamydomonas* and *Chloromonas*, described as typical algae in temperate forest soils (Neustupa, 2001 and references therein), and members of presumably non-photosynthetic Trebouxiophyceae (*Prototheca*-relative OTUs).

Epiphytic substrates as tree bark are less humid and dry over longer periods (arid) than soil (Hoffmann, 1989). Therefore, members of Trebouxiophyceae which exhibit various adaptions to desiccation like mucilage (e.g. *Coccomyxa*), thick cell walls (e.g. *Apatococcus*) and stress metabolites like polyols, may have more competitive advantages on tree bark (Gustavs et al., 2011; Holzinger and Karsten, 2013). The PCA analysis (Fig. 6) indicated an obvious overlap between tree bark and soil algal communities (altogether ten trebouxiophycean OTUs). Two of the most common OTUs on tree bark, *Elliptochloris* OTU 10 and *Apatococcus* OTU 03, were found almost equally frequent in the soil as well. A few OTUs which have clear preference to tree bark were also found in the soil, but mostly with less frequency there, e.g. *Coccomyxa* OTU 07 and OTU 08, (Table 2) Conversely, *Desmococcus* was retrieved from the soil of four sampling sites, but observed on tree bark only in two samples. The occurrence of Trebouxiophyceae in soil could be explained by lichens which grow on the soil surface and on tree bark as well, e.g. photobionts like *Dictyochloropsis*, *Elliptochloris*, *Myrmecia* and *Pseudococcomyxa* (Hoffmann et al., 2007; Khaybullina et al., 2010). In addition, dispersal by wind and rain water may transport biofilm algae from tree bark into soil.

Trends with respect to forest type

The green algal communities indicated some tendencies with respect to different host species trees and forest types. The PCA analysis (Fig. 5) exhibited differences between the algal composition on tree bark of spruce and beech trees. Additionally, less OTU richness in samples of spruce bark could be observed, i.e. an average of 6.3 different OTUs in sample from spruce (three samples) and 7.6 OTUs from beech (five samples) tree bark. These findings could be explained by different structures/microhabitats of tree bark, like roughness (Lüttge and Büdel, 2010), water availability or light (UV irradiance). The tendency of less OTU richness of needle-leaved tree barks compared to those of broadleaved were already observed but exhibited low significance (Kulichová et al., 2014).

The soil algal communities exhibited no obvious differences in OTU richness between beech and spruce forest, i.e. a total of 22 OTUs were detected in soil of spruce forests and 24 OTUs in beech. However, former studies observed in soils of needle-leaved forests a lower diversity and abundance of soil algae than in broadleaved forest, presumably because they are highly acidic and nutrient-poor (Lukešová and Hoffmann, 1996; Neustupa, 2001, and references therein; Novakovskaya and Patova, 2008). Some OTUs indicated preferences to sampling sites of different management types. Remarkably one of the most abundant OTUs, *Prototheca*-relative OTU 26, was not discovered in the unmanaged beech samples, while it was present in spruce and beech age class forests. OTUs with preference to beech forest were represented by *Prototheca*-relative OTU 29 and *Desmococcus*, which were not retrieved from spruce forest samples.

Obvious differences in the OTU composition between the soil types, Leptosol (AEW2, AEW7 and AEW9) and Cambisol, could not be observed. Interestingly, the OTU richness of unmanaged forest sampling sites was the lowest one, especially of chlorophycean OTUs One can expect there the specialist taxa. On the other hand the age class forest sites were higher diverse and there generalists could be supposed. A former study observed less algal density in old forest soils compared to deforested soils, it could be related to low light intensity due to high litter and cover (Lukešová and Hoffmann, 1996). Our findings may also explained by low light or water capability.

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Supplementary Material chapter 5

Table S1. Green algal OTUs recovered from soil and tree bark, their genus assignment, representing clone or isolated strain 18S rRNA gene sequence for OTU and their percentage identities calculated from p-distances with closest available relative. An asterisk mark representative full-length sequences in phylogenetic analysis. A 'P' indicates representative partial sequences (588-866 nts long).

Table S1. Green algal OTUs recovered from soil and tree bark, their genus assignment, representing clone or isolated strain 18S rRNA gene sequence for OTU and their percentage identities calculated from p-distances with closest available relative. An asterisk mark representative full-length sequences in phylogenetic analysis. A 'P' indicates representative partial sequences (588-866 nts long).

OTU Name	OTU No.	Representative clone/isolate	Acc. No.	Substrate	Similarity (%)	Next neighbouring available sequence (acc. no.)	Habitat of references
Trebouxiophyceae							
Apatococcus	OTU 01	clone AEW6B_K237*	KP081318	soil	99.9, 99.8, 99.3	JX169826, JX169825, JX169829	plastic, tree bark
		clone AEW7R_K37*	KP081319	bark	99.1, 99.1, 99.0	JX169826, JX169825, JX169829	plastic, tree bark
	OTU 02	clone AEW4B_K311^{P}	KP081320	soil	99.8	JX169829	plastic
	OTU 03	clone AEW2R_K261^{P}, clone AEW2B_K100^{P}	KP081321, KP081322	bark, soil	99.3	JX169829	plastic
	OTU 04	clone AEW6R_K299^{P}	KP081323	bark	99.4, 99.3	JX169826, JX169825,	plastic, tree bark
	OTU 05	clone AEW1B_K142^{P}	KP081324	soil	99.8, 99.6, 98.3	JX169825, JX169826, JX169829	tree bark, plastic
		clone AEW7R_K193^{P}	KP081325	bark	99.9, 99.7	JX169825, JX169826, JX169829	tree bark, plastic
Asterochloris	OTU 25	clone BOAEW3R1_K5^{P}	KP081358	bark	99.7	AB080310	lichen
Auxenochlorella	OTU 54	isolate SAG 2478*	KP081390	soil	96.3, 99.9	X56101, AM260449^{P}	plant sap, photobiont
Chlorella	OTU 59	isolates KP09AW8003^{P}, (KP09AW8002^{P})	KP081408	bark	99.8	FM205832	freshwater
Chloroidium	OTU 14	clone AEW5R_K23*	KP081343	bark	99.9, 99.3	AB058306, FM946000	unknown, plant sap
		clone AEW2B K61*	KP081344	soil	99.1	AB058306, FM946000	unknown, plant sap
		isolate_LH08AW8042*	KP081400	soil	100	FM946000	plant sap
	OTU 15	clone AEW2B_K102^{P}	KP081345	soil	98.6, 98.5	AB058306, FM946000	unknown, plant sap
Coccomyxa	OTU 06	clone AEW8B_K10^{P}	KP081326	soil	99.4	KF673392	epiphytic
		clone AEW8R_K219^{P}	KP081327	bark	99.6, 99.6, 99.5	AM167525, AJ302939, KF673392	symbiont, endophytic, epiphytic
	OTU 07	clone AEW7R_K95*	KP081328	bark	99.0	KF673392	epiphytic
		clone AEW8R_K237	KP081329	bark	99.4	KF673392	epiphytic
		clone AEW2B_K12^{P}	KP081330	soil	99.5, 99.4	AM981206, KF673392	freshwater, epiphytic
		isolate SAG 2483*	KP081391	soil	100, 100	AM167525, HE586508	endophytic, tree bark
	OTU 08	clone AEW2B_K42*	KP081331	soil	97.4, 97.3	HE586505, KM020051	sandstone rocks, soil
		clone AEW7R_K132*	KP081332	bark	97.5, 97.4	HE586505, KM020051	sandstone rocks, soil
	OTU 09	clone AEW6R_K296^{P}	KP081333	bark	96.6	KM020051, HE586505	soil, wet rocks
	OTU 53	isolate LH08AW1017*	KP081392	soil	99.9, 99.8	HE617184, HE586514	acidic mining lake, bark
Desmococcus	OTU 32	clone AEW5R_K28*	KP081367	bark	100	KM020049	Vienna, tree bark
		clone AEW7B_K371*	KP081368	soil	99.8	KM020049	Vienna, tree bark
Dictyochloropsis	OTU 16	clone AEW1B_K153*	KP081346	soil	99.9	GU017662, JQ988930	soil
		clone AEW7R_K99*	KP081347	bark	99.8	GU017662, JQ988930	soil
		isolate LH08AW3050*	JQ988930	soil	100	GU017662	soil
		isolate SAG 2305	GU017658	bark	99.9	GU017662	soil

Table S1. Continued.

OTU Name	OTU No.	Representative clone/isolate	Acc. No.	Substrate	Similarity (%)	Next neighbouring available sequence (acc. no.)	Habitat of references
	OTU 17	clone AEW1R_K251[P]	KP081348	bark	98.8	GU017659	tree bark
	OTU 18	clone AEW1R_K245*	KP081349	bark	99.3, 99.3	GU017665, GU017660	lichen
	OTU 19	clone BOAEW3R1_K1[P]	KP081350	bark	98.2	GU017659, GU017671	tree bark, epiphytic
Diplosphaera	OTU 36	clone AEW9B_K461*	KP081374	soil	99.5	KF673370, AJ416105, KM020116	from moss antarctica, lichen, bog
Elliptochloris	OTU 10	clone AEW2B_K19	KP081334	soil	99.5, 99.4	FR865675, FJ648518	soil antarctic, tree bark japan
		clone AEW4B_K321*	KP081335	soil	99.6, 99.5	FR865675, FJ648518	soil antarctic, tree bark japan
		clone AEW7R_K168	KP081336	bark	99.8	FR865675,FJ648518	soil antarctic, tree bark japan
		clone AEW7R_K114*	KP081337	bark	99.3	FR865675,FJ648518	soil antarctic, tree bark japan
	OTU 12	clone AEW4B_K318[P]	KP081340	soil	99.9	FJ648515	rooftile
	OTU 13	clone AEW8R_K241*	KP081341	bark	97.7, 97.3	HQ317310, FR865675	cryptoendolithic, soil
		clone AEW7B_K389*	KP081342	soil	97.2	HQ317310, FR865675	cryptoendolithic, soil
Hemichloris	OTU 11	clone AEW2R_K266[P]	KP081338	bark	99.6	KM020164	endolithic
		clone AEW3B_K198[P]	KP081339	soil	99.0, 98.5	FJ648518, KM020164	tree bark, endolithic
Lobosphaera	OTU 20	clone AEW2B_K6*	KP081351	soil	99.9	Z47209	lichen
		isolate_LH08AW3064*	KP081398	soil	100, 99.8	HQ902935, EU878372	unknown, soil
Neocystis	OTU 37	isolate SAG 2480*	KP081393	soil	99.9	KM020044, JQ920367	soil
		clone AEW4BD_K25[P]	KP081375	soil	99.0, 98.9	KF144212, LH08AW8001	freshwater
Pseudochlorella	OTU 35	clone AEW7R_K174*	KP081371	bark	99.8	AM422985, KM020138	soil, freshwater
		clone AEW7R_K093	KP081372	bark	99.6	AM422985	soil
		clone AEW7R_K128	KP081373	bark	99.6	AM422985	soil
Prototheca	OTU 31	clone AEW3B_K185*	KP081366	soil	94.6	AB470468	human skin
Prototheca -relative	OTU 26	clone AEW2B_K16	KP081359	soil	84.7	KJ616755	
		clone AEW5B_K253*	KP081360	soil	84.8	KJ616755	
		clone AEW5B_K265	KP081361	soil	84.9	KJ616755	
	OTU 27	clone AEW3B_K182*	KP081362	soil	84.1	KJ616755	
	OTU 28	clone AEW9B_K453[P]	KP081363	soil	80.9, 80.3	AB183622, KJ616755	
	OTU 29	clone AEW5B_K252*	KP081364	soil	84.6	KJ616755	
	OTU 30	clone AEW7B_K364*	KP081365	soil	84.4	KJ616755	
Stichococcus	OTU 33	clone AEW4B_K337*	KP081369	soil	99.06	EF200531, AJ311637	tree bark, freshwater
	OTU 34	clone AEW8B_K21[P]	KP081370	soil	99.8	AJ311637	freshwater water dish
	OTU 55	isolate SAG 2481*	KP081394	soil	100	AB055866	
		isolates KP09AW1004, (KP09AW1006[P], KP09AW1001[P])	KP081407	bark	100 (99.9)	AB055866	

Table S1. Continued.

OTU Name	OTU No.	Representative clone/isolate	Acc. No.	Substrate	Similarity (%)	Next neighbouring available sequence (acc. no.)	Habitat of references
	OTU 56	isolates SAG 2482*, LH08AW8104	KP081395, KP081396	soil	100	KF144238	freshwater (deinschwangerbach)
	OTU 57	isolate LH08AW8025*	KP081397	soil	100	AY762604	terrestrial facade of building
Trebouxia	OTU 21	clone AEW6R_K315[P]	KP081352	bark	99.7, 99.4	JX391009, EU123942	stone, lichen
	OTU 22	clone AEW6B_K245[P]	KP081353	soil	97.6	Z68700	lichen
		clone AEW6R_K290[P]	KP081354	bark	98.0	Z68700	lichen
	OTU 23	clone AEW6B_K207*	KP081355	soil	99.0, 98.8	JX169846, Z21551	plastic, lichen
		clone AEW7R_K112*	KP081356	bark	98.4, 98.3	JX169846, Z21551	plastic, lichen
	OTU 24	clone AEW6B_K241[P]	KP081357	soil	99.5, 99.3	Z21551, JX169846	lichen, plastic
unident. trebouxiophyte	OTU 38	clone AEW2B_K3[P]	KP081376	soil	97.9, 97.3, 96.3	FJ790649, HE984579, LH08AW3007	
	OTU 40	clone BOAEW3R1_K19[P]	KP081377	bark	94.9	X74000	
	OTU 58	isolate SAG 2477*	KP081399	soil	96.4	FJ790649	quartz in a Tibet desert
Xylochloris	OTU 39	clone AEW2R_K255*	JQ988938	bark	98.9, 98.5, 96.7	JQ988942, JQ988940, EU105209	soil, tree bark
		isolate SAG 2382*	JQ988942	soil	99.6, 96.6	JQ988940, EU105209	soil, tree bark
Chlorophyceae							
Bracteacoccus	OTU 48	clone AEW4B_K327*	KP081385	soil	99.7	AF513376, JQ259925	desert
Chlamydomonas	OTU 51	clone AEW3B_K167*	KP081388	soil	98.1	FR865595	freshwater
Chlamydomonadales	OTU 42	clone AEW6B_K216*	KP081379	soil	97.3	FR865553	freshwater
		isoate SAG 2476*	KP081401	soil	97.7	FR865553	freshwater
Chlorococcum	OTU 41	clone AEW5B_K298*	KP081378	soil	99.7, 99.4	EF024027, JN903985, GU117588	soil, snow
Chlorococcum	OTU 62	isolate SAG 2479*	KP081402	soil	99.8	JN968585	
		isolate LH08AW5111	KP081403	soil	99.8	JN968585	
		isolate LH08AW5107	KP081404	soil	99.7	JN968585	
Chloromonas	OTU 44	clone AEW2B_K74[P]	KP081381	soil	99.4	FR865528	freshwater
	OTU 45	clone AEW1B_K125*	KP081382	soil	99.4	AJ410447	snow
	OTU 46	clone AEW2B_K54[P]	KP081383	soil	98.9	AB906343	snow, Mt. Hakkoda, Japan
	OTU 47	clone AEW5B_K281[P]	KP081384	soil	99.2	AF514402, AF514400	snowfields
Coelastrella	OTU 60	isolate LH08AW4118[P]	KP081406	soil	100	AB012846	
Dictyochloris	OTU 52	clone AEW6B_K249[P]	KP081389	soil	98.8	AF367861	soil
Jenufa	OTU 61	isolate LH08AW8098*	KP081405	soil	100	AB257660, JQ988927	rock, stone (bunker)
		isolate SAG 2383*	JQ988933	soil	99.9	AB257660, JQ988927	rock, stone (bunker)
Microglena	OTU 49	clone AEW4B_K326[P]	KP081386	soil	99.9	FR854374	freshwater
Tetracystis	OTU 43	clone AEW4B_K340*	KP081380	soil	98.7, 98.5	EF023701, KM020030	soil
	OTU 50	clone AEW2B_K17*	KP081387	soil	99.8	KM020018	soil

Chapter 6:

Diversity of green algae in grassland and forest soils under different management types - a culture-independent approach

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Manuscript

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Author contributions to the work:
Performed the experiments: CH.
Analyzed data: CH.
Statistical support (multivariate analysis): LH.
Wrote the paper: CH, TF.
Conceived and designed the experiments: TF, CH.

Abstract

The diversity of soil algae from 57 defined research plots of three German regions, Schwäbische Alb, Schorfheide-Chorin and Hainich-Dün, were investigated using a culture-independent cloning and sequencing approach. The taxonomic composition of green algal communities and their changes in relation to region identity, different land-use types and different management regimes and intensities were determined. For the molecular phylogenetic identification 18S rRNA gene clone libraries were established directly from 27 grassland and 30 forest soils. A total of 145 operational taxonomic units (OTUs) was recovered, representing mainly the green algal classes Trebouxiophyceae and Chlorophyceae. Multivariate statistics pointed out significantly different green algal composition in grassland and forest soils, best explained by soil pH. Grassland soils exhibited a higher diverse community than forest soils. The most widely distributed OTUs, with particular dominance in grassland, were affiliated to *Chloroidium*, *Stichococcus*, *Muriella*, *Tetracystis* and *Bracteacoccus*, whereas OTUs related to *Apatococcus*, *Desmococcus* and *Prototheca*-relatives were dominant or exclusively discovered in forest soils. In relation to management types and intensities, soils in unmanaged forest sites indicated markedly less OTU richness than in managed age class forests. However, this was not evident for the grassland plots. The soil algal community structure exhibited also differences between forest types. Whereas beech and spruce forest soils were dominated by Trebouxiophyceae, in pine forest soils Chlorophyceae were mainly retrieved. In addition a seasonal comparison of selected grassland and forest plots was performed. The algal communities within one sampling site indicated more similar structures and seasonal variations have no significant effect.

Keywords: green algae, soil, land-use, environmental sample, cloning, 18S rRNA gene.

Introduction

Soil habitats are most important non-aqueous ecosystems for algal communities; they are present in relatively large amounts in the top soil layer (Shields and Durrell, 1964; Ruble and Davis, 1988; Wöhler et al., 1988; Zenova et al., 1995; Kabirov and Gaisina, 2009). Soil algae comprise eukaryotic representatives like green algae, diatoms, xanthophytes and eustigmatophytes and prokaryotes like cyanobacteria (Metting, 1981; Starks et al., 1981; Hoffmann, 1989). They have beneficial functions in soil, e.g. provide organic matter as food source, perform nitrogen fixation, and interact with higher plants (Metting, 1981; Starks et al., 1981). These phototrophic communities are among the first organisms colonizing bare soils or disturbed sites and promote soil stability and constitute water-stable aggregates to prevent erosion (Johansen, 1993; Büdel et al., 2014). Environmental factors which influence algae growth and community structures in soil are light, moisture, temperature, availability of nutrients, pH and soil type (Shields and Durrell, 1964; Metting, 1981; Tester and Morris, 1987; Hoffmann, 1989).

Since soil algae are prevalent in topsoil, they are among the first soil organisms exposed to environmental changes and hence integrate environmental and seasonal changes over relatively short periods of time (Bérard et al., 2005). Soil algae are especially sensitive towards soil solution chemistry and thus are promising as potential bioindicators for soil quality (Bérard et al., 2004, 2005; Zancan et al., 2006). Algae are influenced by synthetic pesticides and pollutants; as well the supply rate of N and P strongly affects the growth of algae (Metting, 1981; Smith et al., 1999; Temraleeva et al., 2011). However, despite the vital ecological and physiological importance and numerous studies on soil algae (e.g. Shields and Durrell, 1964; Metting, 1981; Starks et al., 1981; Lukešová, 2001; Hoffmann et al., 2007; Bakieva et al., 2012), it is still difficult to draw general conclusions on the diversity of the flora and their influence on ecosystem functions (Zancan et al., 2006). The latter authors studied changes in algal communities in different agroecosystems treated by tillage and pesticides. They mainly concluded that the structure of soil algal communities was obviously affected more by land-use intensities rather than physicochemical parameters of the soil. Their estimates of mean abundances were based on colony counts in algal cultures which did not allow discriminating at the genus and species levels. Most studies on soil algae, especially related to land-use, have been based on morphological observations. Molecular approaches are still increasing (Hallmann et al., in prep. and references therein), up to now there are merely case studies or the overall

eukaryotic community going not into further detail (Bérard et al., 2005; Lentendu et al., 2014). In the present study the green algal communities in German grassland and forest soils were investigated by a culture-independent approach. In this large scale study 18S rRNA gene sequence comparison and phylogenetic methods lead to a reliable and unambiguous re-identification on soil algae. Here we present the taxonomic composition of soil algal communities and their changes in relation to region identity, different land-use types and different management intensities, whereas to take into account the physico-chemical parameters of soil.

Material and Methods

Sampling site, sampling, soil properties, cultivation

This study was conducted within the large-scale and long-term project 'Biodiversity Exploratories' (www.biodiversity-exploratories.de). The soil samples were collected from three areas in Germany: the UNESCO Biosphere Reserve Schwäbische Alb (AEG, grassland; AEW, forest), a limestone middle mountain range area in South-western Germany, the Hainich-Dün region including the National Park Hainich (HEG, HEW) in central Germany and the UNESCO Biosphere Reserve Schorfheide-Chorin (SEG, SEW), a glacial landscape in North-eastern Germany (Fischer et al., 2010). The Exploratory Schorfheide-Chorin represents with an annual precipitation of 500-600 nm and an annual average temperature of 8-8.5 °C the driest and warmest region of the three research sites followed by the Hainich-Dün region (500-800 nm and 6.5-8 °C) and Schwäbische Alb (700-1000 nm and 6-7 °C; Fischer et al., 2010). The samples were taken from each region from nine grassland and nine forest plots (except Hainich-Dün with 12 forest plots). Both land-use types comprised different management types and land-use intensities, with triplicates of each management type (Table 1 and 2, Fischer et al., 2010). The sampling was conducted through a joint effort in April and May 2008. In each plot five soil cores were taken from a given area of 20 m x 20 m by a motor-driven soil column cylinder and samples of the same plot and the same soil horizon were pooled (for details see Will et al., 2010). For this study 57 samples from the A-horizon (up to 10 cm) were taken for further analysis and one sample from the B-horizon (plot HEG7). Soil characteristics like pH, organic carbon (OC) and total nitrogen (TN) content were taken from Nacke et al. (2011) and the project database BExIS, measurements were described in Will et al. (2010). Data for total phosphorus (TP) and measurements are described in Alt et al. (2011).

Table 1. Grassland sampling sites of the three German Exploratories. Soil parameters and numbers of the recovered green algal OTUs from the single plots.

Plot	Location latitude,	longitude	Management type	Soil type	pH	OC (g kg^{-1})	Total N (g kg^{-1})	All sequences	Algal sequences	Non algal sequences (*Dileptus* sp.)	No. of algal OTUs
AEG1	48°23'52.8" N	9°20'31.2" E	Fertilized meadow	Leptosol	6.70	77.09	7.58	53	48	5 (3)	20
AEG2	48°22'36.7" N	9°28'22.0" E	Fertilized meadow	Leptosol	6.90	72.25	7.18	52	50	2	22
AEG3	48°24'32.0" N	9°31'56.6" E	Fertilized meadow	Leptosol	6.30	53.74	5.19	28	27	1	12
AEG4	48°22'51.2" N	9°25'8.0" E	Fertilized mown pasture, cattle	Leptosol	5.10	51.61	5.35	47	44	3 (2)	20
AEG5	48°23'45.2" N	9°26'21.1" E	Fertilized mown pasture, cattle/horses	Leptosol	6.40	85.16	7.87	69	62	7 (2)	20
AEG6	48°24'4.5" N	9°26'30.0" E	Fertilized mown pasture, cattle/horses	Leptosol	6.10	68.17	6.67	34	29	5 (1)	17
AEG7	48°23'29.1" N	9°22'36.6" E	Unfertilized pasture, sheep	Leptosol	7.20	40.85	3.65	47	39	8 (1)	11
AEG8	48°25'21.5" N	9°29'31.6" E	Unfertilized pasture, sheep	Leptosol	6.50	81.15	7.41	38	35	3	1
AEG9	48°23'40.8" N	9°30'10.1" E	Unfertilized pasture, sheep	Leptosol	6.70	68.89	5.82	43	41	2	2
HEG1	50°58'17.9" N	10°24'19.3" E	Fertilized meadow	Cambisol	6.60	66.20	6.24	51	50	1	15
HEG2	51°0'2.7" N	10°25'48.0" E	Fertilized meadow	Vertisol	7.10	32.60	3.34	41	32	9	16
HEG3	50°59'53.1" N	10°25'58.6" E	Fertilized meadow	Vertisol	7.20	26.02	2.90	58	57	1	25
HEG4	51°6'47.5" N	10°26'10.3" E	Fertilized mown pasture, cattle	Stagnosol	6.50	66.95	6.02	25	21	4	14
HEG5	51°12'57.2" N	10°19'21.1" E	Fertilized mown pasture, cattle	Stagnosol	6.90	53.41	5.09	57	50	7	26
HEG6	51°12'54.2" N	10°23'28.4" E	Fertilized mown pasture, cattle	Stagnosol	6.00	14.24	1.63	75	71	4	31
HEG7	51°16'25.6" N	10°24'38.5" E	Unfertilized pasture, cattle	Stagnosol	6.90	70.08	6.36	57	54	3	21
HEG8	51°16'16.2" N	10°25'4.6" E	Unfertilized pasture, cattle	Stagnosol	7.00	74.84	6.90	33	24	9	12
HEG9	51°13'26.1" N	10°22'51.3" E	Unfertilized pasture, cattle	Stagnosol	6.60	48.27	4.13	79	59	20	26
SEG1	53°5'14.7" N	13°58'10.7" E	Fertilized meadow	Histosol	7.30	196.33	18.82	51	40	11	16
SEG2	53°5'21.5" N	13°58'48.2" E	Fertilized meadow	Histosol	7.40	120.77	12.32	34	30	4 (1)	17
SEG3	53°6'10.2" N	13°59'8.5" E	Fertilized meadow	Histosol	7.40	91.68	9.35	27	18	9	11
SEG4	53°6'49.5" N	14°0'6.7" E	Unfertilized mown pasture, cattle	Histosol	7.40	135.31	13.57	24	20	4	11
SEG5	53°6'26.8" N	14°0'1.9" E	Unfertilized mown pasture, cattle	Gleysol	7.40	104.50	10.84	41	34	7 (3)	12
SEG6	53°6'13.2" N	13°37'22.2" E	Unfertilized mown pasture, cattle	Histosol	5.20	284.10	23.93	27	22	5 (1)	11
SEG7	53°5'18.2" N	13°58'37.4" E	Unfertilized pasture, cattle	Histosol	7.30	117.43	11.61	53	38	15 (11)	20
SEG8	53°6'50.0" N	14°1'0.5" E	Unfertilized pasture, cattle	Gleysol	7.40	73.16	7.05	46	31	15 (1)	14
SEG9	53°5'53.5" N	13°36'45.2" E	Unfertilized pasture, cattle	Histosol	6.20	229.69	18.69	33	26	7 (1)	9

Table 2. Forest sampling sites of the three German Exploratories. Soil parameters and numbers of the recovered green algal OTUs from the single plots.

Plot	Location latitude,	longitude	Management type	Soil type	pH	OC (g kg^{-1})	Total N (g kg^{-1})	All sequences	Algal sequences	Non algal sequences (*Dileptus* sp.)	No. of algal OTUs
AEW1	48°28'41.1" N	9°20'3.9" E	Spruce age class forest	Cambisol	3.30	64.57	3.97	45	4	41 (36)	3
AEW2	48°22'48.0" N	9°21'5.2" E	Spruce age class forest	Leptosol	4.60	65.19	4.35	102	61	41 (24)	16
AEW3	48°24'44.1" N	9°21'20.1" E	Spruce age class forest	Cambisol	5.00	74.68	5.14	37	33	4 (2)	7
AEW4	48°23'56.8" N	9°14'41.4" E	Beech age class forest	Cambisol	6.40	78.50	6.01	51	38	13 (6)	10
AEW5	48°25'10.6" N	9°24'52.9" E	Beech age class forest	Cambisol	4.50	57.53	4.45	38	36	2 (1)	5
AEW6	48°23'38.6" N	9°26'45.4" E	Beech age class forest	Cambisol	5.40	39.05	3.15	38	30	8	13
AEW7	48°23'46.5" N	9°15'40.9" E	Unmanaged beech forest	Leptosol	4.90	77.62	5.54	39	38	1	4
AEW8	48°22'57.3" N	9°22'56.6" E	Unmanaged beech forest	Cambisol	5.10	105.00	6.77	56	56	-	7
AEW9	48°22'9.6" N	9°24'54.8" E	Unmanaged beech forest	Leptosol	6.40	60.03	4.49	34	29	5 (5)	3
HEW1	51°11'7.3" N	10°19'25.0" E	Spruce age class forest	Stagnosol	6.70	69.14	5.22	46	32	14 (7)	4
HEW2	51°12'37.0" N	10°22'11.8" E	Spruce age class forest	Stagnosol	4.50	50.60	3.12	53	31	22 (10)	8
HEW3	51°16'17.3" N	10°18'37.7" E	Spruce age class forest	Luvisol	3.90	47.74	2.88	45	10	35 (28)	8
HEW4	51°22'11.1" N	10°32'1.5" E	Beech age class forest	Luvisol	6.20	79.44	5.79	3	3	-	1
HEW5	51°15'50.9" N	10°14'25.9" E	Beech age class forest	Luvisol	4.80	61.77	4.94	66	46	20	8
HEW6	51°16'3.8" N	10°14'20.2" E	Beech age class forest	Luvisol	4.20	34.40	2.44	54	26	28 (19)	13
HEW7	51°7'51.9" N	10°23'7.6" E	Beech selection forest	Luvisol	4.40	47.04	3.09	37	22	15 (10)	16
HEW8	51°21'21.8" N	10°31'1.1" E	Beech selection forest	Luvisol	5.30	38.23	2.55	40	17	23 (16)	1
HEW9	51°7'48.9" N	10°22'52.1" E	Beech selection forest	Luvisol	4.10	48.04	3.44	60	30	30 (21)	10
HEW10	51°5'24.6" N	10°27'45.8" E	Unmanaged beech forest	Stagnosol	4.80	67.59	5.09	43	-	43 (39)	0
HEW11	51°6'10.2" N	10°24'3.1" E	Unmanaged beech forest	Luvisol	4.80	58.52	4.45	48	41	7	4
HEW12	51°6'3.1" N	10°27'18.6" E	Unmanaged beech forest	Luvisol	3.90	31.13	2.04	34	28	6 (5)	6
SEW1	52°54'3.4" N	13°50'46.9" E	Pine age class forest	Cambisol	3.60	18.34	0.96	31	28	3 (3)	8
SEW2	52°57'6.2" N	13°46'40.9" E	Pine age class forest	Cambisol	3.80	5.38	0.47	47	35	12 (11)	3
SEW3	52°55'14.6" N	13°38'36.4" E	Pine age class forest	Cambisol	3.20	20.95	1.13	45	19	26 (20)	13
SEW4	52°55'2.4" N	13°50'50.3" E	Beech age class forest	Cambisol	3.20	33.17	1.62	62	28	34 (27)	17
SEW5	53°3' 25.3" N	13°53'7.3" E	Beech age class forest	Cambisol	3.10	29.56	1.62	80	13	67 (50)	6
SEW6	52°54'26.8" N	13°50'30.1" E	Beech age class forest	Cambisol	3.30	31.05	1.79	63	30	33 (22)	14
SEW7	53°6'25.8" N	13°41'39.9" E	Unmanaged beech forest	Cambisol	3.20	24.30	1.49	70	24	46 (43)	8
SEW8	53°11'30.5" N	13°55'49.2" E	Unmanaged beech forest	Albeluvisol	3.10	29.20	1.82	56	12	44 (39)	8
SEW9	53°2'40.3" N	13°48'37.2" E	Unmanaged beech forest	Cambisol	3.00	22.96	1.38	46	8	38 (27)	3

Values for land-use intensity, LUI, were taken from project database BExIS (Fischer et al., 2010) and described in Blüthgen et al. (2012). Additionally 15 soil samples were collected of three selected grassland (HEG7, HEG8 and HEG9) and four forest plots (HEW3, HEW4, HEW5 and HEW10) in the Hainich-Dün area in September 2010 (samples labeled with an S, e.g. HEG7S) and March 2011 (samples labeled with a W, e.g. HEG7W; Table 6). From each plot three samples were taken in the given area of 20 m x 20 m (North-east, South-west and from the center) from the upper 0-3 cm. The three samples from the same plot were pooled to mitigate differences, litter and roots were removed. In addition, of a single plot, HEG7 (March 2011), three individual samples within the same plot, NE, SW and the center, were not mixed, but investigated individually (North-east labeled HEG7NOW; center labeled HEG7MW; South-west labeled HEG7SWW). From two samples (HEG7W and HEW3W, both taken in March 2011) enrichment cultures in liquid media were established (see Hodač et al., in prep.).

DNA extraction, PCR amplification, cloning and sequencing

Genomic DNA from approximately 10 g of soil samples were extracted with the MoBio PowerSoil DNA isolation Kit (MoBio Laboratories Inc. Carlsbad, CA) according to the manufacturer's instructions. For further details and DNA extraction of enrichment cultures see Hallmann et al., 2013a and Hallmann et al., in prep.). Extraction results were evaluated after electrophoresis on a 1 % (w/v) agarose gel. Isolated DNA was stored at -20 °C until further processing. For PCR 18S rRNA genes were first amplified using eukaryotic specific primers 20F and 18L (Hallmann et al., 2013a; Hamby et al., 1988) followed by a second amplification (semi- nested PCR) with the primers 20F and the green algae preferring primer CH1750R (Hallmann et al., 2013a). PCR conditions were as described previously (Hallmann et al., 2013a). In a further approach instead of the reverse primer CH1750R the green algal preferring primer 1650Rmod (5′ TCACCAGCACAYYCAAT 3′) under previously described conditions were used. All PCR products were purified using the Invisorb DNA CleanUp Kit (STRATEC Molecular). Aliquots of 2 µl of the purified amplicons were analysed by electrophoresis on a 1 % (w/v) agarose gel.

Cloning was carried out with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) with TOP 10 chemically competent One Shot *Escherichia coli* cells (Invitrogen), as supplied by the manufacturer. In the plasmid blue/white screening, white *E. coli* colonies containing correct DNA insertions were further identified by direct amplification of the inserted DNA fragment with a vector-specific primer set M13F/M13R (Invitrogen).

Positive clones were cultivated overnight in 2 ml reaction tubes with 1 ml LB medium containing 100 µg ampicillin. Plasmid DNA was purified with the Invisorb Spin Plasmid Mini Two kit (STRATEC Molecular) and stored at -20 °C. Sequencing reactions were performed with the Dye Terminator Cycle Sequencing v3.1 kit (Applied Biosystems, Darmstadt, Germany) and an ABI Prism 3100 (Applied Biosystems) automated sequencer. All eukaryotic clones were sequenced with the 18S standard sequencing primer 895R, resulting in partial sequences, including the hypervariable regions V2-V4 (Hallmann et al., 2013a).

Phylogenetic and statistical analysis

The sequences were edited and assembled using SeqAssem (Hepperle, 2004). Sequences shorter than 400 bp were excluded from further analysis. The remaining sequences were compared with available sequences in NCBI by BLASTN (Altschul et al., 1990; http://www.ncbi.nlm.nih.gov/). Non-algal sequences were excluded from further analysis. Next relative sequences were imported into the ARB program (Ludwig et al., 2004; http://www.arb-home.de) to determine preliminary phylogenetic affiliations. Potential chimeras were checked by Bellerophon (Huber et al., 2004); in addition, the first and the last 300 bp of putative chimeras were compared with similar rRNA gene sequences in NCBI. Chimeric sequences were excluded from the data set. Rarefaction curves and operational taxonomic units (OTUs) were calculated with MOTHUR version 1.13.0 (Schloss et al., 2009). OTUs were defined on the basis of ≥ 98 % sequence similarity (Hallmann et al., 2013a). Representative sequences of almost each OTU were selected and sequenced over almost entire length (≥ 1639 bp) with standard sequencing primers for phylogenetic analysis. For phylogenetic analyses, two alignments of almost full length 18S rRNA gene sequences were performed using MAFFT, version 6 (Katoh and Toh, 2008). The alignment of Trebouxiophyceae included 164 sequences and 1821 positions (892 variables, 619 parsimony informative positions), the dataset of Chlorophyceae contained 167 sequences and 1798 positions (848 variable, 593 parsimony informative). Complete rRNA gene sequences were subjected to phylogenetic analyses using the maximum likelihood (ML) method by RAxML (Stamatakis et al., 2008), in conjunction with the GTR+Γ+I model with 100 bootstrap replicates. In addition, Bayesian posterior probabilities (MB) were calculated with MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001). Two parallel Markov chain Monte Carlo (MCMC) runs for two million generations each

with one cold and three heated chains were conducted using the GTR+Γ+I model, with trees sampled every 100 generations.

The overall similarity of clone libraries from grassland and forest soils was compared based on a presence/absence matrix of the OTU occurrence. The binary matrix was used for Canonical Correspondence Analysis (CCA). To correlate the green algal OTUs with soil properties Spearman's rank correlation were computed. In order to identify clusters of OTU occurring at similar sampling plots, we conducted Principal Components Analysis (PCA) of two data subsets representing clone libraries from grassland and forest sites. To assess sampling and seasonal effects of the OTUs a Detrended Correspondence Analysis (DCA) were accomplished of a selected dataset (Table 6). All statistical analyses were computed with the program PAST 2.17c (Hammer et al., 2001).

Results and Discussion

Overall diversity and richness

For this study samples from topsoil (A-horizon) were collected from 27 grassland and 30 forest sites from three regions in Germany, Schorfheide-Chorin, Hainich-Dün and Schwäbische Alb, representing a north-south gradient. Both land-use types comprise different management types and intensities like fertilized and intensely managed grasslands to unfertilized grazed pastures and spruce or pine managed age class forests to unmanaged beech forests (Tables 1 and 2).

Using a culture-independent cloning and sequencing approach a total of 1860 green algal 18S rRNA gene sequences were analysed after quality and chimera check. A number of 145 green algal OTUs were recovered based on 98 % sequence similarity. To determine the clone library coverage for the algal sequences, rarefaction curves were calculated for grassland and forest sites of each Exploratory (Fig. 1a and b). The curves for the grassland samples reached nearly full coverage, whereas the forest samples were not saturated. The OTU richness of the single sampling sites differed between one and 31 green algal OTUs (Table 1 and 2). Except for one forest plot (HEW10), out of 43 sequences no green algal sequence could be retrieved. The green algal (Chlorophyta) preferring primer (CH1750R) resulted also in non-algal sequences. For sample HEW10 a high fraction (39 sequences) of an alveolate-related 18S rRNA gene sequence, a representative full length clone (1573 nucleotides) exhibited 97 % blast similarity to *Dileptus microstoma* HM581676, was retrieved. Dileptids are ciliates known from terrestrial habitats like soil and leaf litter

(Vďačný and Foissner, 2008). Presumably this ciliate was abundant at this sampling site and masked the green algae there. Altogether 71.3 % of the non-algal sequences at all forest sites belonged to this *Dileptus* related sequences, this high abundance resulted also in less green algal sequences for these sites, however, only 15.8 % of the non-algal sequences in grassland soils were affiliated to this ciliate.

The retrieved 145 OTUs were distributed over the green algal classes Chlorophyceae (71 OTUs), Trebouxiophyceae (70 OTUs), Ulvophyceae (3 OTUs) and Pedinophyceae (1 OTU). The largest amount of sequences belonged to the Trebouxiophyceae (66.5 %), 32.2 % of all retrieved sequences belonged to Chlorophyceae, while a small fraction of 1.3 % of the clones were affiliated to Ulvophyceae and Pedinophyceae (Table 3). The molecular approach enabled the identification of the soil algae down to the generic or even species level. Therefore a representative full length sequence of almost each OTU was established for the phylogenetic analysis or the next available relative sequence (as determined from BLAST queries) was used (Table 4). According to the phylogenetic analysis the trebouxiophycean OTUs were distributed over the Chlorellales (including e.g. the *Chlorella*-, *Marvania*-, *Auxenochlorella*- and *Protoheca*-clade, Fig. 2), the *Choricystis*/*Botryococcus*-clade, including species of *Coccomyxa* and *Elliptochloris*, the *Prasiola*-, *Trebouxia*- and the *Watanabea*-clades *sensu* Leliaert et al. (2012) and Friedl and Rybalka (2012) and several independent single lineages (Fig. 2). The Trebouxiophyceae comprises mainly terrestrial species, adapted to drier habitats like soil/desert and air exposed substrates, furthermore lichen symbionts and freshwater species (Friedl and Büdel, 2008; Friedl and Rybalka, 2012; Hallmann et al., 2013a; Fučíková et al., 2014).

The chlorophycean OTUs were phylogenetically (Fig. 3) mainly distributed over Chlamydomonadales (motile representatives with flagella) and Sphaeropleales (includes also non-motile algae) and a few representatives of Chaetopeltidales and Chaetophorales, including also freshwater and terrestrial species (Lewis and McCourt, 2004; Friedl and Rybalka, 2012). The class of Ulvophyceae includes species from marine, freshwater and terrestrial habitats, the latter were distributed in lineages like Ulotrichales and Scotinophaerales (Friedl and O'Kelly, 2002; Friedl and Rybalka, 2012; Škaloud et al., 2013). The one retrieved relative of Pedinophyceae, OTU 142 was closely related to *Pedinomonas minor*, was also known from soil (Ettl and Gärtner, 1995).

Most groups and genera identified at the here described sampling sites were well-known from temperate soil habitats like e.g. *Bracteacoccus*, *Chlamydomonas*, members of Chlorellales, *Chlorococcum*, *Chloromonas*, *Coccomyxa*, *Elliptochloris*, *Leptosira*,

Myrmecia, *Stichococcus*, and *Tetracystis* (e.g. Reisigl, 1964; Metting, 1981; Ettl and Gärtner, 1995; Lukešová and Hoffmann, 1996; Lukešová, 2001; Neustupa and Škaloud, 2004; Zancan et al., 2006; Hoffmann et al., 2007; Khaybullina et al., 2010; Bakieva et al., 2012). A few of the detected OTUs, but with less clone numbers, were so far identified from the Tropics, Antarctica or deserts e.g. *Leptochlorella, Xerochlorella* and *Xylochloris* (Němcová et al., 2011; Hodač et al., 2012; Neustupa et al., 2013; Fučíková et al., 2014). Some of the here retrieved OTUs could not be further identified because of taxonomic problems and several of the OTUs may represent still new undescribed species or even new genera e.g. *Prototheca* relative OTUs 31-36, Chlorellales relative OTUs 43, 46 and 49 (Fig. 2). In a parallel approach the same 57 soil samples were analysed after classical enrichment of soil algae in liquid media and establishment of isolates (Hodač et al., in prep). The microscopic evaluation and sequencing of the obtained isolates allowed a reliable description of new species.

The occurrence of the OTUs were distributed as follows, 4.1 % of the OTUs were retrieved frequently from more than 15 sampling sites out of 56, 15.2 % were found at 9-14 sites, 46.2 % at 2-8 sites and 34.5 % of the OTUs were recovered only at a single sampling site, these 50 OTUs included 32 singleton OTUs (Table 3). Six OTUs were frequently received and presented in relatively high sequence numbers (Table 3). The most abundant OTU across all samples was *Chloroidium* OTU 15, phylogenetically affiliated to *Chloroidium saccharophilum*, detected in 25 of 56 plots and representing with 7.8 % the most frequent algal sequences (Fig. 2, Table 4). The genus *Chloroidium*, in earlier studies assigned to *Chlorella*, is widespread in different kinds of substrates like in freshwater and terrestrial, including soil (Lukešová and Hoffmann, 1996; Zancan et al., 2006; Darienko et al., 2010). OTUs which were as well recovered in high frequency were phylogenetically assigned to: *Stichococcus minutus* OTU 54 (21/56 plots), *Muriella* sp. OTU 42 and *Tetracystis vinatzeri* OTU 118 each found at 17 plots and *Tetracystis* sp. OTU 84 and *Bracteacoccus minor* OTU 107, both occurred at 15 plots (Table 3, Table 4, Fig. 2, Fig. 3).

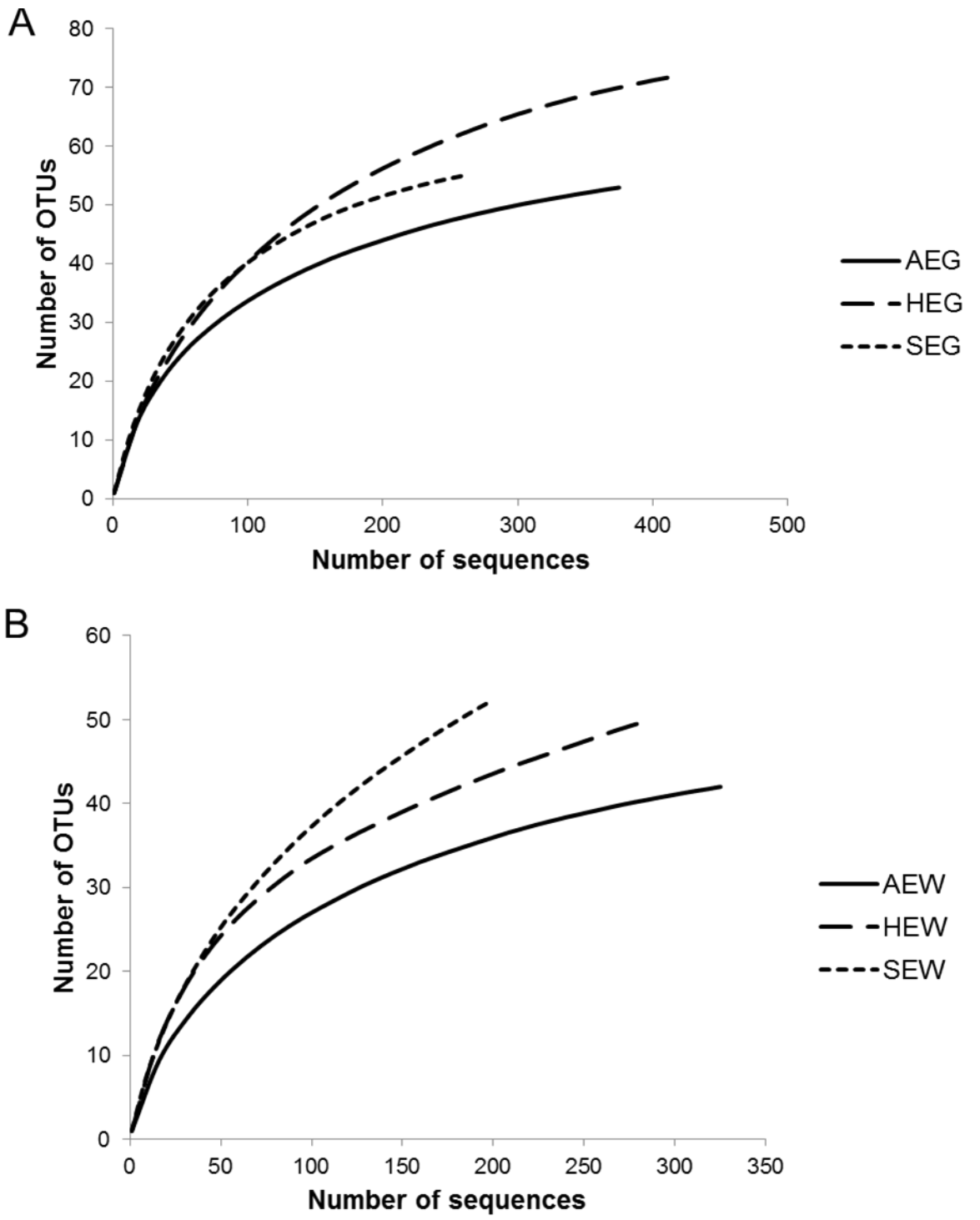

Fig. 1. Rarefaction curves determined for 18S rRNA gene clone libraries. The OTUs include the green algal sequences from (A) grassland and from (B) forest soil samples of the three German research sites Schwäbische Alb (AEG, AEW), Hainich-Dün (HEG, HEW) and Schorfheide-Chorin (SEG, SEW).

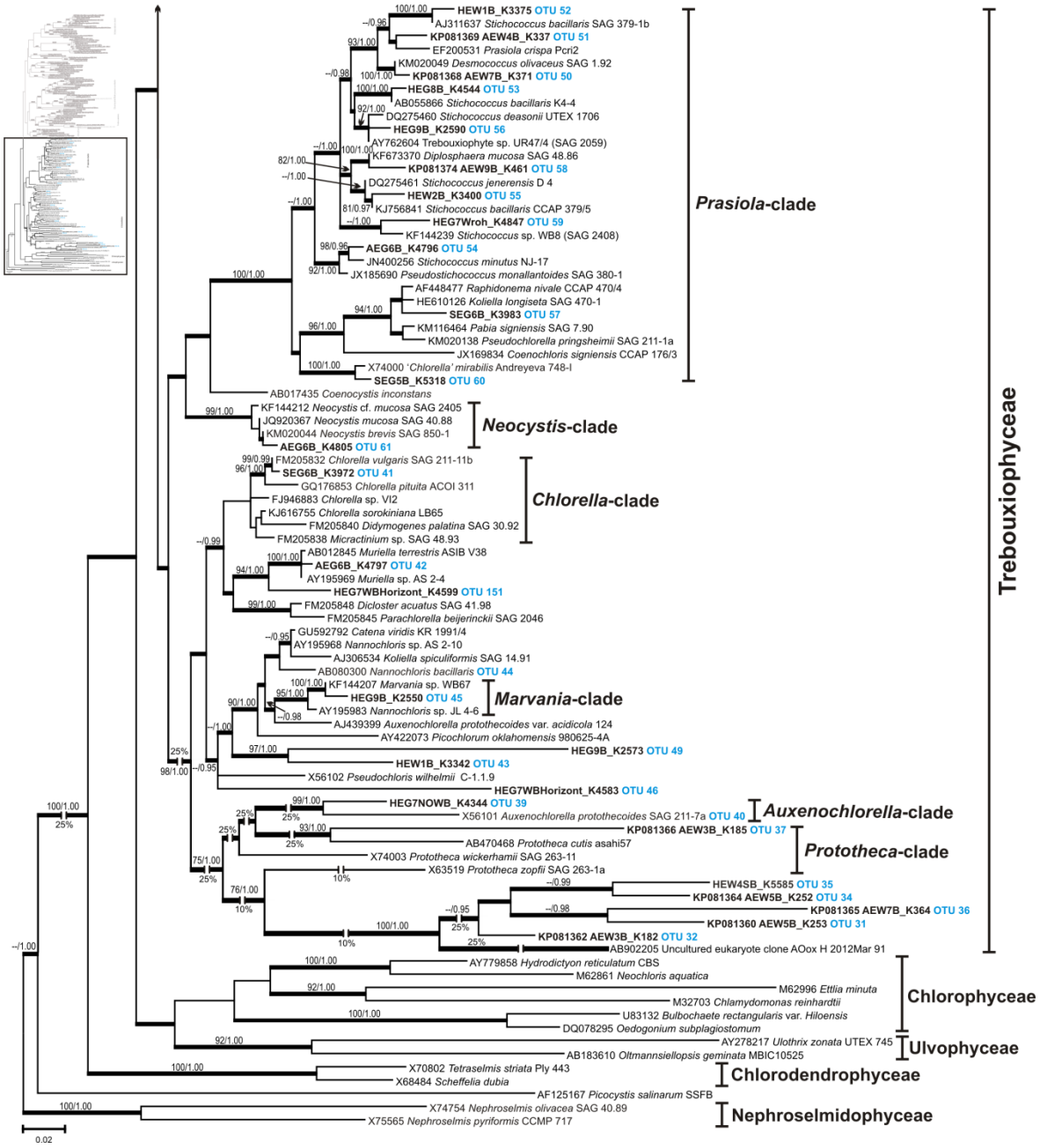

Fig. 2. Maximum likelihood tree (RAxML) of **Trebouxiophyceae** based on 18S rRNA gene sequences from German grassland and forest soils with two members of Nephroselmidophyceae as outgroup taxa. Sequences in bold represent full 18S rRNA gene sequences of this study, blue labels mark representatives of the recovered OTUs. Thick lines indicate internal nodes that were received in maximum likelihood and Bayesian trees. Support values given as ML (Maximum Likelihood; >75 % bootstrap values) /MB (MrBayes; >0.95 Bayesian posterior probabilities).

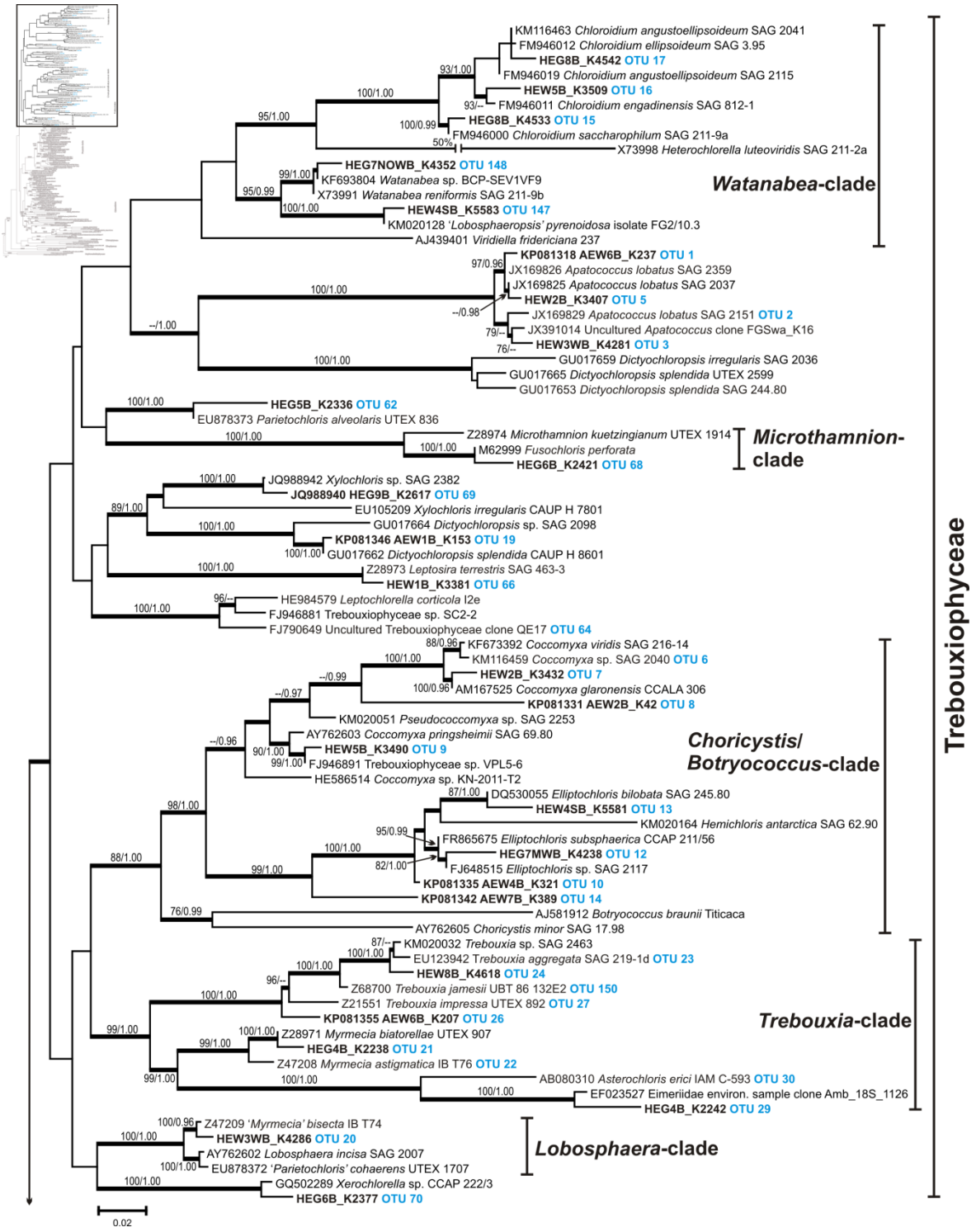

Fig. 2. Continued.

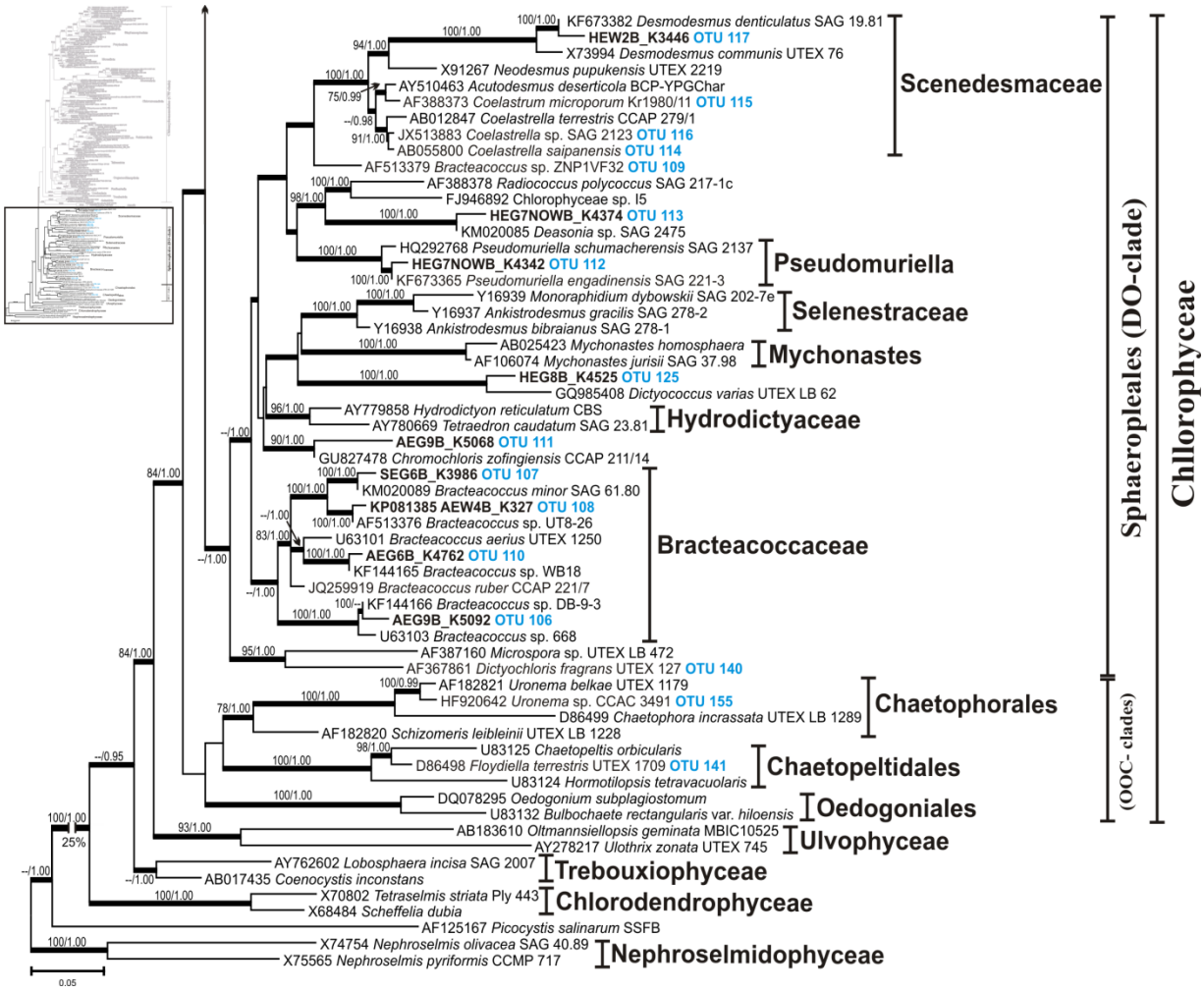

Fig. 3. Maximum likelihood tree (RAxML) of **Chlorophyceae** based on 18S rRNA gene sequences from German grassland and forest soils with two members of Nephroselmidophyceae as outgroup taxa. Sequences in bold represent full 18S rRNA gene sequences of this study, blue labels mark representatives of the recovered OTUs. Thick lines indicate internal nodes that were received in maximum likelihood and Bayesian trees. Support values given as ML (Maximum Likelihood; >75 % bootstrap values) /MB (MrBayes; >0.95 Bayesian posterior probabilities).

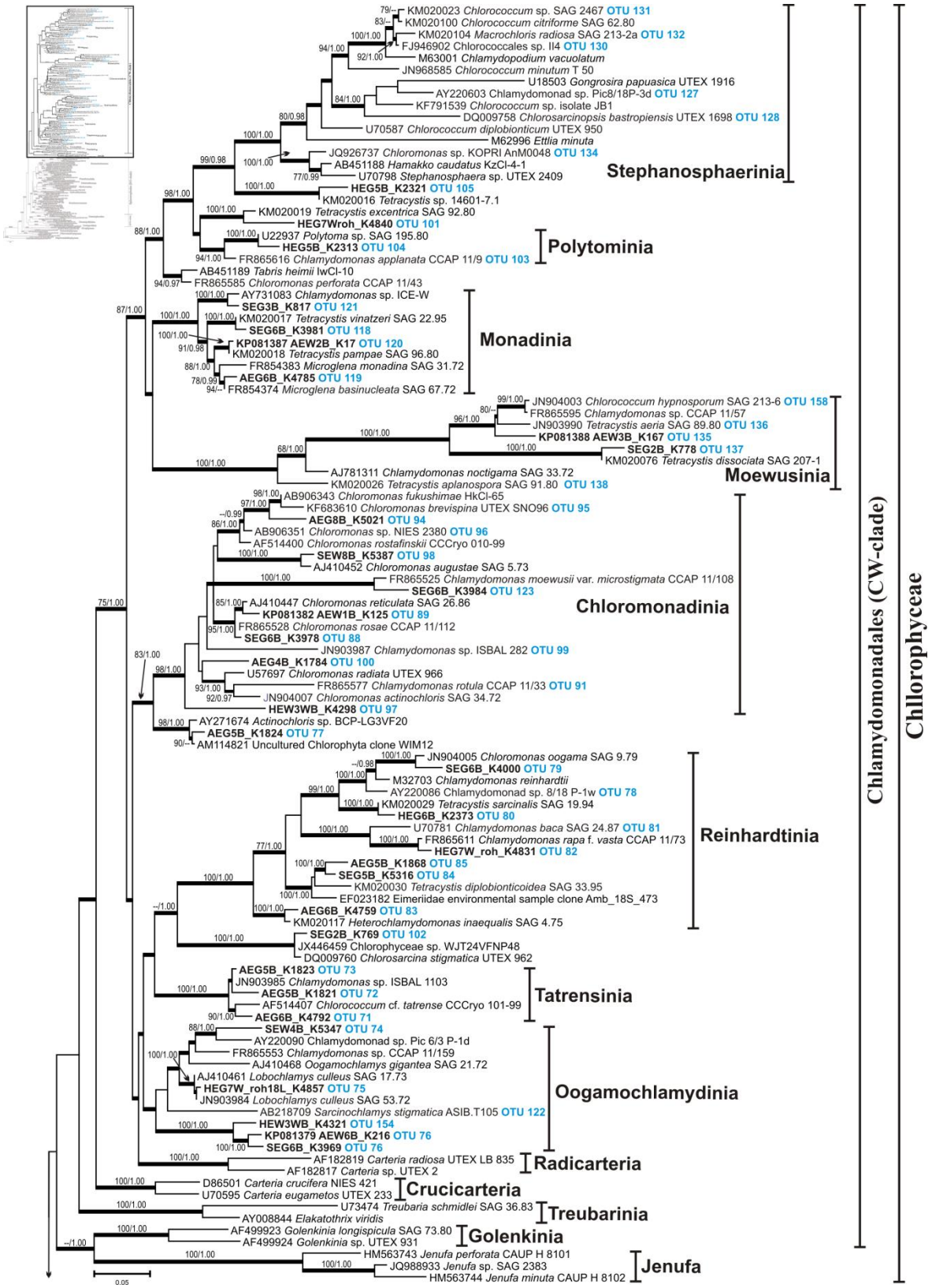

Fig. 3. Continued.

Table 3. Distribution of 145 green algal OTUs in the 18S rRNA gene clone libraries from 27 grassland and 30 forest soils of the three German research sites. Numbers indicated frequencies of plots where the OTUs were recovered. The OTUs belong to the green algal classes of Trebouxiophyceae, Chlorophyceae, Ulvophyceae and Pedinophyceae.

		grassland plots			forest plots			
OTU name	**OTU no.**	**AEG (9)**	**HEG (9)**	**SEG (9)**	**AEW (9)**	**HEW (12)**	**SEW (9)**	**total no. of sequences**
Trebouxiophyceae								
Apatococcus	OTU 1	-	-	-	1	1	-	3
	OTU 2	-	-	-	1	1	1	4
	OTU 3	-	-	-	3	2	3	14
	OTU 4	-	-	-	-	-	1	8
	OTU 5	-	-	-	1	1	1	9
Asterochloris	OTU 29	-	3	-	-	-	1	6
	OTU 30	-	1	-	-	-	-	4
Auxenochlorella	OTU 39	3	1	-	-	-	2	8
	OTU 40	5	2	5	-	1	-	30
Chlorella	OTU 41	1	4	4	-	-	-	14
Chlorellales relative	OTU 43	1	2	-	-	1	-	12
	OTU 46	-	2	1	-	-	-	4
	OTU 49	1	4	-	-	-	-	6
'Chlorella' mirabilis	OTU 60	1	-	2	-	-	-	5
Chloroidium	OTU 15	7	8	5	1	2	2	146
	OTU 16	3	6	1	1	1	1	52
	OTU 17	3	7	2	-	-	1	44
	OTU 18	-	1	-	-	-	-	1
Coccomyxa	OTU 6	-	-	-	1	3	1	12
	OTU 7	-	1	-	1	2	2	17
	OTU 8	-	-	-	2	-	2	5
	OTU 9	1	3	1	-	2	1	13
Desmococcus	OTU 50	-	1	-	4	3	3	57
Dictyochloropsis	OTU 19	-	-	-	2	3	-	11
Diplosphaera	OTU 58	1	3	1	1	1	1	19
Elliptochloris	OTU 10	-	-	-	5	1	5	22
	OTU 11	-	-	-	1	1	1	5
	OTU 12	3	3	-	1	-	2	17
	OTU 13	-	1	-	-	-	-	2
	OTU 14	-	-	-	3	-	-	20
Fuscochloris	OTU 68	-	4	-	-	-	-	8
Helicosporidium relative	OTU 38	-	-	-	-	1	-	1
Koliella	OTU 57	3	2	6	-	-	-	36
Leptochlorella	OTU 64	-	-	-	1	-	-	1
Leptosira	OTU 66	1	8	2	-	1	-	46
	OTU 67	-	2	-	-	-	-	2
Lobosphaera	OTU 20	2	3	-	1	1	2	20

Table 3. Continued.

OTU name	OTU no.	grassland plots AEG (9)	HEG (9)	SEG (9)	forest plots AEW (9)	HEW (12)	SEW (9)	total no. of sequences
Marvania	OTU 45	3	4	2	-	1	-	16
Muriella	OTU 42	6	9	1	-	1	-	50
Myrmecia	OTU 21	1	2	-	-	-	-	3
	OTU 22	1	-	-	-	-	-	1
Nannochloris	OTU 44	-	2	-	-	-	-	2
Neocystis	OTU 61	4	1	-	1	2	1	14
Parietochloris	OTU 62	-	1	-	-	2	-	5
Prasiola	OTU 51	3	-	-	1	-	-	19
Prototheca	OTU 37	-	-	-	1	1	-	6
Prototheca relative	OTU 31	-	-	-	4	3	-	88
	OTU 32	-	-	-	2	3	-	23
	OTU 33	-	-	-	3	-	-	10
	OTU 34	-	-	-	4	4	4	143
	OTU 35	-	-	-	-	1	-	1
	OTU 36	-	-	-	1	-	-	3
Stichococcus	OTU 52	2	2	3	1	2	2	31
	OTU 53	2	2	-	-	2	-	12
	OTU 54	6	8	4	-	2	1	50
	OTU 55	-	4	3	-	2	-	17
	OTU 56	1	1	1	-	-	1	8
	OTU 59	-	1	-	-	-	-	1
Trebouxia	OTU 23	-	-	1	-	-	-	1
	OTU 24	-	-	-	-	2	3	27
	OTU 25	-	-	-	1	-	-	1
	OTU 26	-	-	-	1	-	-	2
	OTU 27	-	-	-	1	1	-	4
	OTU 28	-	-	-	-	1	-	1
Unidentified trebouxiophyte	OTU 47	-	-	-	-	-	1	1
	OTU 48	-	1	-	-	-	-	2
	OTU 63	1	-	-	-	-	-	1
	OTU 65	-	1	-	-	-	-	1
Xerochlorella	OTU 70	1	3	-	-	-	-	6
Xylochloris	OTU 69	2	1	-	-	-	-	3
Chlorophyceae								
Actinochloris	OTU 77	1	-	1	-	-	1	4
Bracteacoccus	OTU 106	2	5	-	-	-	1	28
	OTU 107	6	4	4	-	-	1	21
	OTU 108	2	2	3	2	1	2	23
	OTU 109	-	1	-	-	-	-	2
	OTU 110	3	2	2	-	-	-	13
Chlamydomonas	OTU 72	1	-	-	-	1	-	4
	OTU 73	1	3	-	-	-	1	9
	OTU 78	-	-	-	-	1	-	1

Table 3. Continued.

OTU name	OTU no.	grassland plots AEG (9)	HEG (9)	SEG (9)	forest plots AEW (9)	HEW (12)	SEW (9)	total no. of sequences
	OTU 81	-	1	-	-	-	-	2
	OTU 82	-	-	2	-	-	-	5
	OTU 91	-	-	-	-	-	1	3
	OTU 99	-	-	-	-	-	1	1
	OTU 103	-	-	2	-	-	-	7
	OTU 121	-	-	1	-	-	-	2
	OTU 123	-	-	1	-	-	-	2
	OTU 124	-	1	-	-	-	-	1
	OTU 135	-	-	-	1	-	2	5
Chlorococcum	OTU 71	4	1	-	1	-	3	19
	OTU 127	1	-	1	-	-	-	2
	OTU 131	-	2	2	-	1	-	10
Chloromonas	OTU 79	-	-	1	-	-	-	1
	OTU 88	2	1	4	2	1	3	26
	OTU 89	-	-	-	1	-	-	1
	OTU 90	-	-	-	-	-	1	7
	OTU 94	1	1	-	2	1	1	46
	OTU 95	-	-	-	-	1	2	4
	OTU 96	-	-	-	1	-	1	2
	OTU 98	-	3	-	-	-	1	7
	OTU 100	1	-	2	-	-	-	3
	OTU 134	-	-	-	-	3	1	12
Chlorosarcina	OTU 102	-	-	5	-	-	-	13
Chlorosarcinopsis	OTU 128	-	-	1	-	-	-	1
Chromochloris	OTU 111	1	-	-	-	-	-	24
Coelastrella	OTU 114	4	6	1	-	-	-	23
Coelastrum	OTU 115	-	1	-	-	-	-	1
	OTU 116	-	1	-	-	-	-	2
Deasonia	OTU 113	-	2	-	-	-	-	2
Desmodesmus	OTU 117	-	-	-	-	1	-	11
Dictyochloris	OTU 140	2	-	-	1	-	-	3
Dictyococcus	OTU 125	1	4	3	-	1	-	17
Floydiella	OTU 141	-	-	1	-	-	-	1
Heterochlamydomonas	OTU 83	2	1	7	-	3	-	24
Lobochlamys	OTU 75	-	-	2	-	-	-	3
Macrochloris	OTU 130	-	1	2	-	-	-	4
	OTU 132	-	-	2	-	-	-	2
Microglena	OTU 119	5	2	2	1	2	-	45
Oogamochlamydinia	OTU 74	-	-	-	-	-	1	1
	OTU 76	-	-	1	2	-	1	11
Polytoma	OTU 104	-	1	-	-	-	-	2
Pseudomuriella	OTU 112	1	1	-	-	-	-	3
Sarcinochlamys	OTU 122	-	-	-	-	-	1	1

Table 3. Continued.

OTU name	OTU no.	grassland plots AEG (9)	HEG (9)	SEG (9)	forest plots AEW (9)	HEW (12)	SEW (9)	total no. of sequences
Tetracystis	OTU 80	-	1	-	-	-	-	1
	OTU 84	3	4	7	1	-	-	32
	OTU 85	1	-	-	-	-	2	9
	OTU 86	-	1	-	-	-	-	1
	OTU 101	1	-	1	-	-	-	5
	OTU 105	-	3	1	-	-	-	7
	OTU 118	6	7	3	-	1	-	50
	OTU 120	1	2	-	1	1	-	7
	OTU 136	-	-	-	-	-	1	1
	OTU 137	-	-	2	-	-	-	3
	OTU 138	-	1	1	-	-	-	3
Unidentified Chlorophyceae	OTU 87	-	-	1	-	-	-	1
	OTU 92	-	-	-	-	-	1	1
	OTU 93	-	-	-	-	-	1	2
	OTU 97	-	-	-	-	-	1	1
	OTU 126	-	-	-	-	1	-	1
	OTU 129	-	-	-	-	-	1	1
	OTU 133	-	-	1	-	-	-	4
	OTU 139	-	-	1	-	-	-	1
Pedinopyhceae								
Pedinomonas	OTU 142	-	-	1	-	-	-	1
Ulvophyceae								
Chlorochytrium	OTU 143	-	1	1	-	-	-	5
Scotinosphaera	OTU 144	-	2	1	-	-	-	5
Pseudendocloniopsis	OTU 145	3	2	2	-	-	1	14

Table 4. Blast identities of representative sequences of the green algal OTUs in the 18S rRNA gene clone libraries from the grassland and forest soils. An asterisk indicates a partial sequence (≥ 506 bp).

OTU name	OTU no.	Representative clone (Acc. no.)	Closest reference sequence	Acc. no.	Blast similatity
Trebouxiophyceae					
Apatococcus	1	AEW6B_K237 (KP081318)	*Apatococcus lobatus* strain SAG 2359	JX169826	99%
	2	SEW4B_K1276*	*Apatococcus lobatus* strain SAG 2151	JX169829	99%
	3	HEW3WB_K4281	Uncultured *Apatococcus* clone FGSwa_K16	JX391014	99%
	4	SEW7B_K1417*	*Apatococcus lobatus* strain SAG 2359	JX169826	99%
	5	HEW2B_K3407	*Apatococcus lobatus* strain SAG 2037	JX169825	99%
	146	HEW3WB_K4325*	Uncultured *Apatococcus* clone FGSwa_K16	JX391014	99%
Asterochloris	30	HEG3B_K2223*	*Asterochloris erici* strain IAM C-593	AB080310	99%
	29	HEG4B_K2242	Eimeriidae environmental clone Amb_18S_1126	EF023527	99%
Auxenochlorella	40	AEG3B_K1692*	*Auxenochlorella protothecoides* strain SAG 211-7a	X56101	99%
	39	HEG7NOWB_K4344	*Auxenochlorella protothecoides* strain SAG 211-7a	X56101	97%
Chlorella	41	SEG6B_K3972	*Chlorella vulgaris* strain SAG 211-11b	FM205832	99%
Chlorellales relative	43	HEW1B_K3342	*Nannochloris* sp. strain AS 2-10	AY195968	97%
	46	HEG7W_BHorizont_K4583	*Chlorella* sp. strain VI2	FJ946883	96%
	49	HEG9B_K2573	*Micractinium* sp. strain SAG 48.93	FM205838	95%
			Nannochloris sp. isolate AS 2-10	AY195968	95%
'Chlorella' mirabilis	60	SEG5B_K5318	*'Chlorella' mirabilis* strain Andreyeva 748-I	X74000	99%
Chloroidium	15	HEG8B_K4533	*Chloroidium saccharophilum* strain SAG 211-9a	FM946000	99%
	16	HEW5B_K3509	*Chloroidium angustoellipsoideum* strain SAG 2115	FM946019	99%
	17	HEG8B_K4542	*Chloroidium angustoellipsoideum* strain SAG 2041	KM116463	99%
	18	HEG7B_K2505*	*Chloroidium angustoellipsoideum* strain SAG 2041	KM116463	98%
Coccomyxa	6	HEW9B_K3122*	*Coccomyxa* sp. strain SAG 2040	KM116459	99%
	7	HEW2B_K3432	*Coccomyxa glaronensis* strain CCALA 306	AM167525	99%
	9	HEW5B_K3490	Trebouxiophyceae sp. strain VPL5-6	FJ946891	99%
			Coccomyxa rayssiae strain UTEX 273	HQ317304	99%
	8	AEW2B_K42 (KP081331)	*Pseudococcomyxa* sp. strain SAG 2253	KM020051	97%
			Coccomyxa sp. strain CCAP 211/97	FN298928	97%
Desmococcus	50	AEW7B_K371 (KP081368)	*Desmococcus olivaceus* strain SAG 35.83	AJ431572	99%
			Desmococcus olivaceus strain SAG 1.92	KM020049	99%
Dictyochloropsis	149	HEG7NB_K3095*	*Dictyochloropsis splendida* strain SAG 244.80	GU017653	98%
	19	AEW1B_K153 (KP081346)	*Dictyochloropsis splendida* strain CAUP H8601	GU017662	99%
Diplosphaera	58	AEW9B_K461 (KP081374)	*Diplosphaera* sp. strain SAG 49.86	KM020116	99%
			Diplosphaera mucosa strain SAG 48.86	KF673370	99%
Elliptochloris	10	AEW4B_K321 (KP081335)	*Elliptochloris subsphaerica* strain CCAP 211/56	FR865675	99%
	11	HEW3B_K5207*	*Elliptochloris subsphaerica* strain SAG 2202	FJ648518	99%
	12	HEG7MWB_K4238	*Elliptochloris* sp. strain SAG 2117	FJ648515	99%
	13	HEW4SB_K5581	*Elliptochloris bilobata* strain SAG 245.80	DQ530055	99%
	14	AEW7B_K389 (KP081342)	*Elliptochloris subsphaerica* strain CCAP 211/56	FR865675	97%
Fuscochloris	68	HEG6B_K2421	*'Characium' perforatum*	M62999	99%
Helicosporidium relative	38	HEW2B_K3394*	*Helicosporidium* sp.	JN869301	89%
Koliella	57	SEG6B_K3983	*Koliella longiseta* strain SAG 470-1	HE610126	99%
			Raphidonema nivale strain CCAP 470/4	AF448477	99%
Leptochlorella	64	AEW2B_K3 (KP081376)*	Uncultured Trebouxiophyceae clone QE17	FJ790649	98%
	153	HEW3Wroh18L_K4936*	*Leptochlorella corticola* strain I2e	HE984579	97%
Leptosira	66	HEW1B_K3381	*Leptosira terrestris* strain SAG 463-3	Z28973	99%
	67	HEG3B_K2234*	*Leptosira terrestris* strain SAG 463-3	Z28973	97%
Lobosphaera /Myrmecia	20	HEW3WB_K4286	*'Myrmecia' bisecta* strain IB T74	Z47209	99%
Marvania	45	HEG9B_K2550	*Marvania* sp. isolate WB67	KF144207	99%
			Nannochloris sp. isolate JL 4-6	AY195983	99%
Muriella	42	AEG6B_K4797	*Muriella* sp. strain AS 2-4	AY195969	99%
	151	HEG7W_BHorizont_K4599	*Muriella* sp. strain AS 2-4	AY195969	99%
Myrmecia	21	HEG4B_K2238	*Myrmecia biatorellae* strain UTEX 907	Z28971	99%
	22	AEG3B_K1690*	*Myrmecia astigmatica* strain IB T76	Z47208	99%
Nannochloris	44	HEG6B_K2436*	*Nannochloris bacillaris*	AB080300	99%
Neocystis	61	AEG6B_K4805	*Neocystis brevis* strain SAG 850-1	KM020044	99%
Parietochloris	62	HEG5B_K2336	*Parietochloris alveolaris* strain UTEX 836	EU878373	99%
Prasiola	51	AEW4B_K337 (KP081369)	*Prasiola crispa* isolate Pcri2	EF200531	99%
Prototheca	37	AEW3B_K185 (KP081366)	*Prototheca cutis* strain asahi57	AB470468	93%

Table 4. Continued.

OTU name	OTU no.	Representative clone (Acc. no.)	Closest reference sequence	Acc. no.	Blast similatity
Prototheca relative	31	AEW5B_K253 (KP081360)	Uncultured eukaryote clone AOox_H_2012Mar_91	AB902205	90%
			Chlorella sorokiniana strain LB65	KJ616755	84%
	32	AEW3B_K182 (KP081362)	Uncultured eukaryote clone AOox_H_2012Mar_91	AB902205	91%
	33	AEW9B_K453 (KP081363)*	Uncultured eukaryote clone AOox_H_2012Mar_91	AB902205	89%
	34	AEW5B_K252 (KP081364)	*Chlorella pituita* strain ACOI 856	FM205856	84%
	35	HEW4SB_K5585	*Chlorella* sp. strain YEL	DQ377322	84%
	36	AEW7B_K364 (KP081365)	*Chlorella* sp. strain YEL	DQ377322	83%
Stichococcus	52	HEW1B_K3375	*Stichococcus bacillaris* strain SAG 379-1b	AJ311637	99%
	53	HEG8B_K4544	*Stichococcus bacillaris* strain K4-4	AB055866	99%
	54	AEG6B_K4796	*Pseudostichococcus monallantoides* strain SAG 380-1	JX185690	99%
			Stichococcus minutus strain NJ-17	JN400256	99%
	55	HEW2B_K3400	*Stichococcus bacillaris* strain CCAP 379/5	KJ756841	99%
	56	HEG9B_K2590	*Stichococcus bacillaris* isolate FG2/4.2	KM020048	99%
			Trebouxiophyte sp. isolate UR47/4 (strain SAG 2059)	AY762604	99%
	59	HEG7W_roh_K4847	*Stichococcus* sp. isolate WB8	KF144239	99%
Trebouxia	23	SEG1B_K719*	*Trebouxia aggregata* strain SAG 219-1d	EU123942	99%
	24	HEW8B_K4618	*Trebouxia* sp. strain SAG 2463	KM020032	99%
	26	AEW6B_K207 (KP081355)	*Trebuxia impressa* strain UTEX 892	Z21551	99%
	27	AEW6B_K241 (KP081357)*	*Trebuxia impressa* strain UTEX 892	Z21551	99%
	28	HEW6B_K2964*	*Trebuxia impressa* strain UTEX 892	Z21551	99%
	150	HEW3W1650R_K5103*	*Trebouxia jamesii* strain UBT-86.132E2	Z68700	99%
	25	AEW6B_K245 (KP081353)*	*Trebuxia jamesii* strain UBT-86.132E2	Z68700	97%
Unidentified trebouxiophyte	47	SEW3B_K1229*	*Neocystis brevis* strain SAG 850-1	KM020044	97%
	48	HEG6B_K2432*	*'Myrmecia' bisecta* strain IB T74	Z47209	96%
	63	AEG4B_K1786*	*Parietochloris alveolaris* strain UTEX 836	EU878373	96%
	65	HEG5B_K2333*	*'Chlorella' mirabilis* strain Andreyeva 748-I	X74000	96%
	147	HEW4SB_K5583	*'Lobosphaeropsis' pyrenoidosa* isolate FG2/10.3	KM020128	99%
	152	HEG7NB_K3092*	*Coenocystis inconstans*	AB017435	98%
			Neocystis cf. *mucosa* strain SAG 2405	KF144212	98%
Watanabea	148	HEG7NOWB_K4352	*Watanabea* sp. strain BCP-SEV1VF9	KF693804	99%
Xerochlorella	70	HEG6B_K2377	*'Dictyosphaerium'* sp. strain CCAP 222/3	GQ502289	99%
Xylochloris	69	HEG9B_K2617 (JQ988940)	*Xylochloris* sp. strain SAG 2382	JQ988942	99%
Chloropyhceae					
Actinochloris	77	AEG5B_K1824	Uncultured Chlorophyta clone WIM12	AM114821	99%
			Actinochloris sp. isolate BCP-LG3VF20	AY271674	99%
Bracteacoccus	106	AEG9B_K5092	*Bracteacoccus* sp. isolate DB-9-3	KF144166	99%
	107	SEG6B_K3986	*Bracteacoccus minor* strain SAG 61.80	KM020089	99%
	108	AEW4B_K327 (KP081385)	*Bracteacoccus* sp. strain UT8-26	AF513376	99%
	109	HEG7B_K2473*	*Bracteacoccus* sp. strain ZNP1VF32	AF513379	99%
	110	AEG6B_K4762	*Bracteacoccus* sp. isolate WB18	KF144165	99%
Chlamydomonas	72	AEG5B_K1821	*Chlamydomonas* sp. strain ISBAL 1103	JN903985	99%
	73	AEG5B_K1823	*Chlamydomonas* sp. strain ISBAL 1103	JN903985	99%
	78	HEW7B_K3264*	Chlamydomonad sp. isolate Pic 8/18 P-1w	AY220086	99%
	81	HEG3B_K2224*	*Chlamydomonas baca* strain SAG 24.87	U70781	99%
	82	HEG7Wroh_K4831	*Chlamydomonas rapa* f. *vasta* strain CCAP 11/73	FR865611	99%
	91	SEW3B_K1234*	*Chlamydomonas rotula* strain CCAP 11/33	FR865577	99%
	99	SEW3B_K1231*	*Chlamydomonas* sp. strain ISBAL 282	JN903987	98%
			Chloromonas carrizoensis strain SAG 46.72	AJ410446	98%
	103	SEG4B_K873*	*Chlamydomonas applanata* strain CCAP 11/9	FR865616	99%
	121	SEG3B_K817	*Chlamydomonas* sp. strain ICE-W	AY731083	99%
	123	SEG6B_K3984	*Chla. moewusii* var. *microstigmata* strain CCAP 11/108	FR865525	99%
	124	HEG9B_K2566*	*Chla. moewusii* var. *microstigmata* strain CCAP 11/108	FR865525	98%
	135	AEW3B_K167 (KP081388)	*Chlamydomonas* sp. strain CCAP 11/57	FR865595	98%
Chlorococcum	71	AEG6B_K4792	*Chlorococcum* cf. *tatrense* strain 101-99	AF514407	99%
	127	AEG2B_K1613*	Chlamydomonad sp. strain Pic8/18P-3d	AY220603	99%
	131	HEW3SB_K4453*	*Chlorococcum* sp. strain SAG 2467	KM020023	99%
	156	HEW3SB_K4467*	*Chlorococcum* sp. strain SAG 2467	KM020023	99%
	158	HEW3SB_K4438*	*Chlorococcum hypnosporum* strain SAG 213-6	JN904003	99%
Chloromonas	79	SEG6B_K4000	*Chloromonas oogama* strain SAG 9.79	JN904005	99%
	88	SEG6B_K3978	*Chloromonas rosae* strain CCAP 11/112	FR865528	99%
	89	AEW1B_K125 (KP081382)	*Chloromonas reticulata* strain SAG 26.86	AJ410447	99%

Table 4. Continued.

OTU name	OTU no.	Representative clone (Acc. no.)	Closest reference sequence	Acc. no.	Blast similatity
	90	SEW1B_K1115*	*Chloromonas actinochloris* strain SAG 34.72	JN904007	98%
	94	AEG8B_K5021	*Chloromonas fukushimae* strain HkCl-65	AB906343	98%
	95	SEW8B_K5393*	*Chloromonas brevispina* strain UTEX SNO96	KF683610	99%
	96	SEW6B_K1390*	*Chloromonas* sp. strain NIES-2380	AB906351	99%
	98	SEW8B_K5387	*Chloromonas augustae* strain SAG 5.73	AJ410452	99%
	100	AEG4B_K1784	*Chloromonas rosae* strain CCAP 11/112	FR865528	98%
	119	AEG6B_K4785	*Chloromonas subdivisa* strain SAG 67.72	FR854374	99%
	134	HEW9B_K3104*	*Chloromonas* sp. strain KOPRI AnM0048	JQ926737	99%
Chlorosarcina	102	SEG2B_K769	*Chlorosarcina stigmatica* strain UTEX 962	DQ009760	99%
	122	HEW3SB_K4423*	*Chlorosarcina stigmatica* strain ASIB.T105	AB218709	99%
Chlorosarcinopsis	128	SEG8B_K1031*	*Chlorosarcinopsis bastropiensis* strain UTEX 1698	DQ009758	99%
Chromochloris	111	AEG9B_K5068	*Chromochloris zofingiensis* strain CCAP 211/14	GU827478	99%
Coelastrella	114	HEG7NOWB_K4333*	*Coelastrella saipanensis*	AB055800	99%
Coelastrum	115	HEG9B_K2615*	*Coelastrum microporum* strain Kr1980/11	AF388373	99%
	116	HEG6B_K2443*	*Coelastrella* sp. strain SAG 2123	JX513883	99%
Deasonia	113	HEG7NOWB_K4374	*Deasonia* sp. strain SAG 2475	KM020085	99%
Desmodesmus	117	HEW2B_K3446	*Desmodesmus denticulatus* strain SAG 19.81	KF673382	99%
Dictyochloris	140	HEW3SB_K4449*	*Dictyochloris fragrans* isolate UTEX_127	AF367861	99%
Dictyococcus	125	HEG8B_K4525	*Dictyococcus varians* strain UTEX LB 62	GQ985408	98%
Floydiella	141	SEG9B_K1066*	*Floydiella terrestris* strain UTEX1709	D86498	98%
Heterochlamydomonas	83	AEG6B_K4759	*Heterochlamydomonas inaequalis* strain SAG 4.75	KM020117	99%
Macrochloris	132	SEG3B_K803*	*Macrochloris radiosa* strain SAG 213-2a	KM020104	99%
	130	SEG6B_K3982*	Chlorococcales sp. strain Il4	FJ946902	99%
Oogamochlamydinia	75	HEG7Wroh_K4857	*Chlamydomonas 'reinhardtii'* strain SAG 53.72	JN903984	99%
			Lobochlamys culleus strain SAG 17.73	AJ410461	99%
	74	SEW4B_K5347	Chlamydomonad sp. isolate Pic 6/3 P-1d	AY220090	98%
	76	SEG6B_K3969	*Chlamydomonas* sp. strain CCAP 11/159	FR865553	97%
	76	KP081379 AEW6B_K216	*Chlamydomonas* sp. strain CCAP 11/159	FR865553	97%
	154	HEW3WB_K4321	*Chlamydomonas* sp. strain CCAP 11/159	FR865553	97%
Polytoma	104	HEG5B_K2313	*Polytoma* sp. strain SAG 195.80	U22937	99%
Pseudomuriella	112	HEG7NOWB_K4342	*Pseudomuriella engadinensis* strain SAG 221-3	KF673365	99%
Tetracystis	80	HEG6B_K2373	*Tetracystis sarcinalis* strain SAG 19.94	KM020029	99%
	84	SEG5B_K5316	*Tetracystis diplobionticoidea* strain SAG 33.95	EF023182	99%
	85	AEG5B_K1868	*Tetracystis diplobionticoidea* strain SAG 33.95	KM020030	98%
	86	HEG9B_K2580*	*Tetracystis diplobionticoidea* strain SAG 33.95	KM020030	98%
	101	HEG7Wroh_K4840	*Tetracystis excentrica* strain SAG 92.80	KM020019	98%
	105	HEG5B_K2321	*Tetracystis* sp. isolate 14601-7.1	KM020016	99%
	118	SEG6B_K3981	*Tetracystis vinatzeri* strain SAG 22.95	KM020017	99%
	120	AEW2B_K17 (KP081387)	*Tetracystis pampae* strain SAG 96.80	KM020018	99%
	136	SEW1B_K1107*	*Tetracystis aeria* strain SAG 89.80	JN903990	99%
	137	SEG2B_K778	*Tetracystis dissociata* strain SAG 207-1	KM020076	99%
	138	SEG2B_K762*	*Tetracystis aplanospora* strain SAG 91.80	KM020026	98%
Unidentified Chlorophyceae	92	HEG7WB_K5556*	*Chloromonas rosae* strain CCAP 11/112	FR865528	98%
	87	SEG9B_K1052*	*Chloromonas perforata* strain CCAP 11/43	FR865585	96%
	93	SEW3B_K1205*	*Chloromonas rosae* strain CCAP 11/112	FR865528	97%
	97	HEW3WB_K4298	*Chloromonas rosae* strain CCAP 11/112	FR865528	97%
	126	HEW7B_K3266*	*Bracteacoccus ruber* strain CCAP 221/7	JQ259919	96%
	129	SEW3B_K1195*	*Chlorococcum diplobionticum* strain UTEX 950	U70587	97%
	133	SEG8B_K1007*	*Chlorococcum* sp. isolate JB1	KF791539	97%
	139	SEG7B_K987*	*Tetracystis aplanospora* strain SAG 91.80	KM020026	95%
	157	HEG7Wroh_K4814*	*Chlorococcum citriforme* strain SAG 62.80	KM020100	96%
Uronema	155	HEW5WB_K4505*	*Uronema* sp. strain CCAC 3491	HF920642	99%
Pedinopyhceae					
Pedinomonas	142	SEG2B_K772*	*Pedinomonas minor* strain SAG 1965-3	HE610132	99%
Ulvophyceae					
Chlorochytrium	143	HEG9B_K2561*	*Chlorochytrium willei* strain ACOI 251	HE860259	99%
Scotinosphaera	144	HEG9B_K2533	*Scotinosphaera lemnae* strain CAUP H5303a	HE860257	99%
Pseudendocloniopsis	145	HEG7Wroh_K4859	*Pseudendocloniopsis botryoides* isolate D2-6-1A	KF144213	99%
			Pseudendocloniopsis botryoides strain CCAP 465/1	FR865755	99%
Printzina	159	HEW3W1650R_K5099*	*Printzina* cf. *lagenifera* UNA00068183	FJ532301	100%

Overall diversity in the study regions

Altogether for the Exploratory Hainich-Dün 704 clones were retrieved, for the Schwäbische Alb 700 and for the region Schorfheide-Chorin 456 clones were recovered. With 95 OTUs and an average of 13.3 OTUs for each plot, the Hainich-Dün region turned out to be the most diverse Exploratory, followed by Schorfheide-Chorin with 93 OTUs (an average of 11.2 OTUs) and Schwäbische Alb with the lowest diversity of 79 OTUs (an average of 10.7 OTUs). Hainich-Dün region and Schwäbische Alb exhibited plots with highest OTU richness. Five of the most diverse plots with 21-31 OTUs were located in Hainich-Dün, four plots representing 20-22 OTUs in Schwäbische Alb, whereas in the Schorfheide-Chorin region only one plot with 20 OTUs was found. Overall the three Exploratories shared 42 OTUs (29 %), OTUs exclusively retrieved in the regions Schorfheide-Chorin, Hainich-Dün and Schwäbische Alb yielded in 19.3 %, 17.2 % and 8.3 %. Compared to OTU richness the region Hainich-Dün and Schwäbische Alb indicated the highest similarity, sharing 57 OTUs, while compared to Schorfheide-Chorin the region Hainich-Dün exhibited 55 OTUs and Schwäbische Alb only 52 OTUs. Soils differed with respect to type, pH, total organic carbon and total nitrogen content (Table 1 and 2). Whereas the Hainich-Dün region and Schwäbische Alb were predominated by soils with higher clay content, Schorfheide-Chorin was dominated by sandy soils. Since clayey soils retain more water than the quickly drying sandy soils, tendencies of wider algal distribution in clay than in lighter or sandy soils were reported (Shields and Durrell, 1964; Lukešová and Hoffmann, 1996).

The Principal Components Analysis (PCA) showed that differences in the composition of green algae between the three Exploratories are more obvious in grassland (Fig. 4) than in forest soils (Fig. 5). The green algal communities in grassland plots of Hainich-Dün region and Schwäbische Alb were more similar with each other, whereas those from the Schorfheide-Chorin Exploratory were more dissimilar from the former two. However, this was not evident at the forest plots (Fig. 5). Though these analyses indicated some tendencies, because low explanatory validation of the total variance was obtained (axis were supported between 9 % and 14 %).

With respect to soil algal communities the green algal class Trebouxiophyceae were dominant in the Hainich-Dün region and Schwäbische Alb with 56 and 50 OTUs, while 36 and 28 OTUs were affiliated to Chlorophyceae. However in Schorfheide-Chorin most OTUs (53) belonged to Chlorophyceae and even 36 OTUs were assigned to Trebouxiophyceae. This finding was particularly evident for the grassland soils.

Chlorophyceae dominated here in all but one (SEG5) plot. Accordingly, the number of trebouxiophycean OTUs was much lower compared to the grassland sites of the other region; 33 OTUs Chlorophyceae and 18 OTUs Trebouxiophyceae were obtained (Table 5). In the Canonical Correspondence Analysis (CCA) the OTU composition of the Schorfheide-Chorin grassland samples exhibited a gradient which correspond to total nitrogen (TN) and organic carbon (OC), here the sampling sites SEG5 and SEG8 showed the greatest differences in OTU composition to SEG6 and SEG9 (Fig. 6).

The Exploratory Schorfheide-Chorin, with many wetlands, is situated at the lowest altitude with 3-140 m a.s.l. of the three investigated regions (Fischer et al., 2010). In the grassland plots of Schorfheide-Chorin occur two different soil types, sandy mineral soil (Gleysol) at the sampling sites SEG5 and SEG8 and drained peat soil (Histosol) with high OC and TN concentrations (Table 1). The drained peat soils were affected by a varying groundwater table, also showed by high Ellenberg values for soil moisture, and in winter and early spring these sites were often flooded (Müller et al., 2012; Herold et al., 2014). The moist to flooded grassland soils in Schorfheide-Chorin were dominated by Chlorophyceae, this class inhabiting many motile species, e.g. like *Heterochlamydomonas* OTU 83 at seven out of nine SEG sites or *Chloromonas* OTU 88 in four and the *Chlamydomonas* OTUs 82, 103, 121, 123 exclusively found at these grassland sites, this condition was more comfortable for motile algae (Hunt et al., 1979). At these sampling sites the number of trebouxiophycean OTUs were lower than in the other regions, most aerophytic algae belonged Trebouxiophyceae and seem to be more adapted to dryer habitats like e.g. building stone and tree bark (Karsten et al., 2007; Hallmann et al., 2013a, 2013b; Kulichová et al., 2014).

Table 5. Distribution of the retrieved green algal OTUs (numbers of OTUs) from grassland (AEG, HEG, SEG) and forest soils (AEW, HEW, SEW) of the three German research sites with respect to the green algal classes.

	Schwäbische Alb		Hainich-Dün		Schorfheide-Chorin	
Green algal class	**AEG**	**AEW**	**HEG**	**HEW**	**SEG**	**SEW**
Trebouxiophyceae	28	30	39	35	18	26
Chlorophyceae	24	12	30	15	33	25
Ulvophyceae	1	-	3	-	3	1
Pedinophyceae	-	-	-	-	1	-
Summary	53	42	72	50	55	52

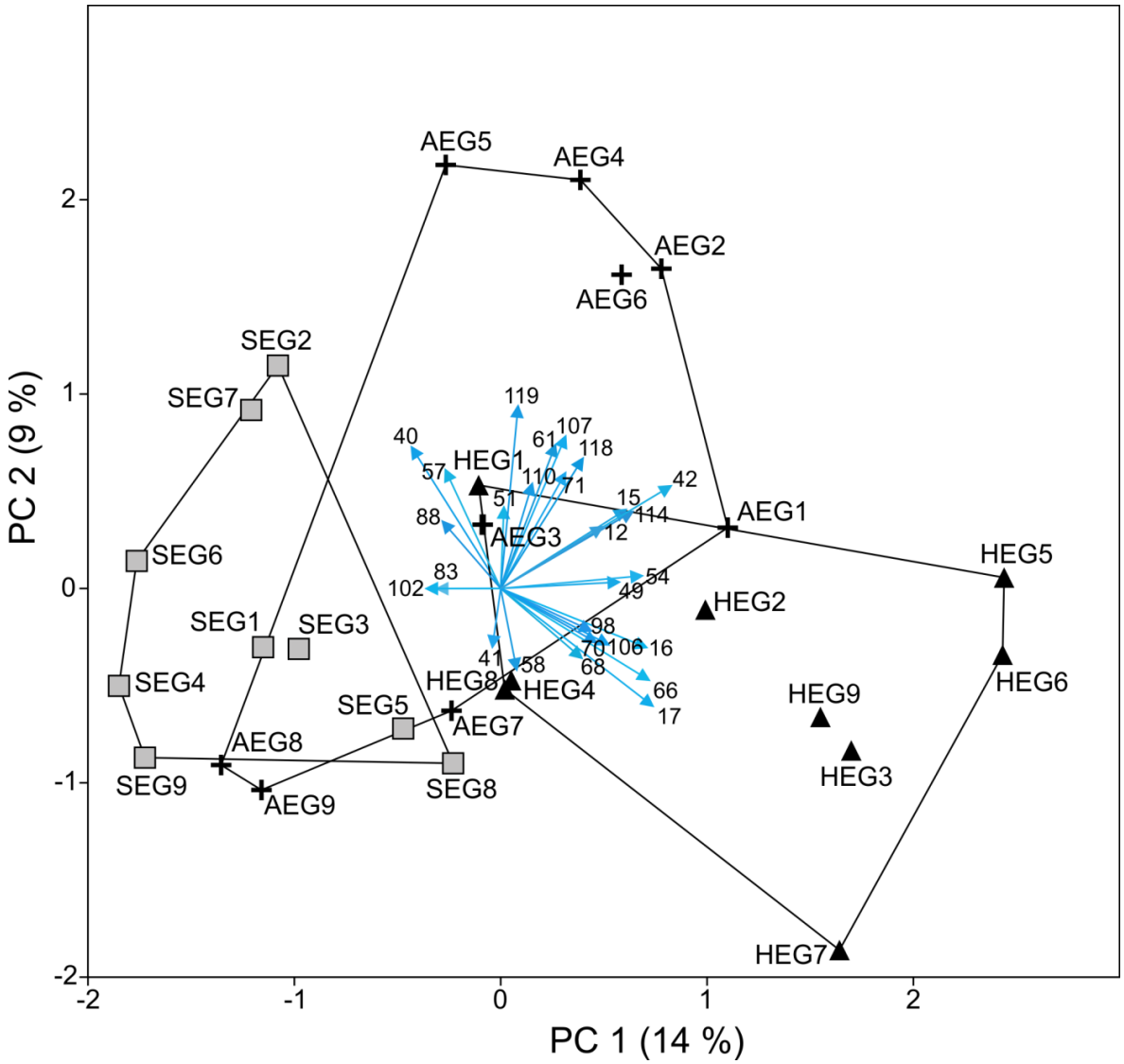

Fig. 4. The Principal Components Analysis (PCA) indicates green algal OTUs (blue arrows) which are responsible for the main differences between grassland soils of the three German research sites. Squares: Schorfheide-Chorin; triangle: Hainich-Dün; cross: Schwäbische Alb. For the species/genus assignments of the OTUs see Table 4.

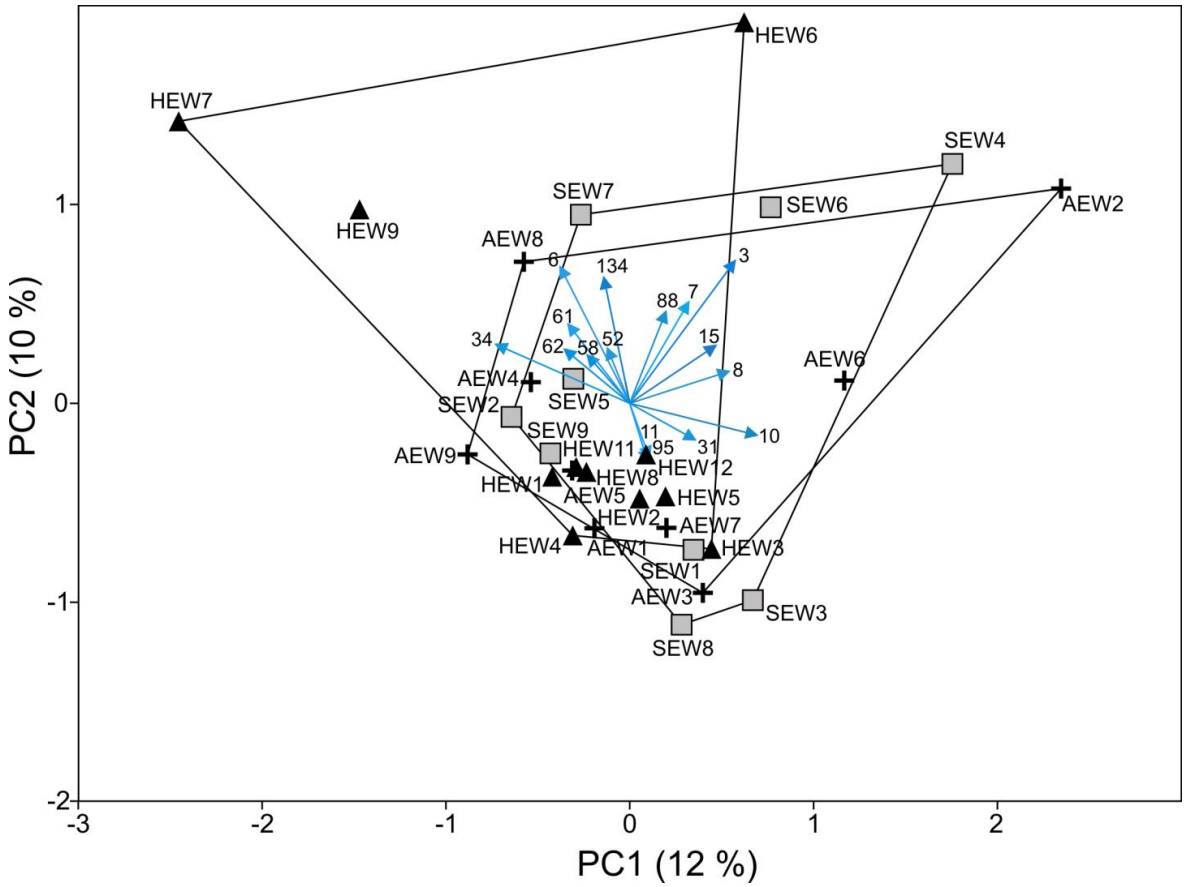

Fig. 5. The Principal Components Analysis (PCA) showing green algal OTUs (blue arrows) which correspond to the main differences between forest soils of the three German research sites. Squares: Schorfheide-Chorin; triangle: Hainich-Dün; cross: Schwäbische Alb. For the species/genus assignments of the OTUs see Table 4.

Differences between land-use type (grassland and forest)

Overall the green algal community of grassland and forest soils exhibited significant differences in OTU richness and composition. In grassland soils a total of 101 OTUs were recovered, dominated by chlorophycean OTUs (53), followed by Trebouxiophyceae (44 OTUs), Ulvophyceae (three OTUs) and the one singleton OTU assigned to Pedinophyceae (Table 3). However in forest soils (89 OTUs) Trebouxiophyceae dominated (50 OTUs), 38 OTUs belonged to Chlorophyceae and one OTU represented Ulvophyceae. To be precise, in 15 out of the 27 grassland plots OTUs mainly assigned to Trebouxiophyceae were retrieved, whereas at 12 sites more chlorophycean OTUs were retrieved, including at eight sites of Schorfheide-Chorin (Table 3). In forest soils the majority of OTUs belonged to Trebouxiophyceae, in 26 of 30 sampling sites, especially in Hainich-Dün and Schwäbische Alb (Table 5). The grassland soils exhibited an obvious higher OTU richness than the forest soils. In grassland soils an average of 16 OTUs per

plot was recovered while in forest soils a much lower diversity was found with an average of 7.8 OTUs. Ten sampling sites in grassland soils exhibited more than 20 OTUs; the maximum was 31 OTUs, thus in forest soils nine plots showed more than 10 OTUs; the maximum was a number of 17 OTUs (Table 1 and 2). It is noticeable that about 11 OTUs were recovered from more than 10 grassland sites, whereas only three OTUs occurred at more than 10 forest sites.

The Canonical Correspondence Analysis CCA (Fig. 6) demonstrated the 56 sampling sites and the distribution due to their OTU composition and the parameter pH, TN and OC. The CCA indicated a clear differentiation between grassland and forest soil algal communities, except of the two sampling sites AEG8 and AEG9 (Fig. 6, Table 3). Beside different factors pH values may best explain the difference between both land-use types at the level of soil algal communities. The pH values in grassland soils range from 5.10-7.40, while pH of forest soils vary from 3.00-6.70, the forest soils of Schorfheide-Chorin indicated acidic soils with the lowest pH values 3.00-3.80 (Table 2). In the CCA (Fig. 6) the data of the X-axis were significantly correlated to pH ($p \leq 0.001$). Additionally to pH, TN and OC also total phosphorus (TP) were tested (data not shown); TP was significantly correlated ($p \leq 0.001$) with pH. The difference of algal composition in grassland and forest soils and the influence of pH on soil alga communities have been shown in previous studies, thus cyanobacteria were preferably in neutral and alkaline soils and therefore less common in forest soils, whereas green algae occur also in acidic soils (Shields and Durell, 1964; Metting, 1981; Hoffmann, 1989; Lukešová and Hoffmann, 1996; Neustupa, 2001). Additionally light radiation may cause the difference between communities in grassland and in forest soils (Hunt et al., 1979).

The grassland and forest soils exhibited different green algal compositions, a total of 46 OTUs (31.7 %) were shared by both land-use types (Table 3). Forty-four OTUs were exclusively retrieved from forest soils e.g. the *Apatococcus* OTUs 1-5, the *Prototheca*-relative OTUs 31-36, *Elliptochloris* OTU 10 and *Dictyochloropsis* OTU 19 whereas a larger number of OTUs (55) were recovered only in grassland soils, e.g. *Chlorella* OTU 41 (phylogenetically assigned to *Chlorella vulgaris*, Fig. 2), *Coelastrella* OTU 144 and *Koliella/Raphidonema* OTU 57 (Table 3). Green algal OTUs with frequent occurrence and obvious preference to grassland soils were: *Auxenochlorella* OTU 40 (phylogenetically assigned to *Auxenochlorella protothecoides*, Fig. 2) *Bracteacoccus* OTU 107, the *Chloroidium* OTUs 15-17, *Heterochlamydomonas* OTU 83, *Leptosira* OTU 66, *Muriella* OTU 42, *Stichococcus* OTU 54 and the *Tetracystis* OTUs 84 and 118. Overall the

members of Chlorellales, except the *Prototheca*-relative OTUs, *Stichococcus*, Sphaeropleales, like *Bracteacoccus*, seem to prefer grassland soils, as well the recovered OTUs assigned to Ulvophyceae. The latter comprise the three OTUs *Chlorochytrium* OTU 143, *Pseudendocloniopsis* OTU 145 and *Scotinosphaera* OTU 144 described from freshwater and terrestrial habitats (Table 4, Friedl and O'Kelly, 2002; Škaloud et al., 2013). Algae with an evidently preference to forest were *Desmococcus* OTU 10 and OTUs assigned to *Coccomyxa* and *Trebouxia.*

Previous studies reported that the algal communities of grassland and forest soils were different, whereas forest soils were less diverse (Metting, 1981; Lukešová, 2001; Neustupa, 2001; Novakovskaya and Patova, 2008). Whereas members of Trebouxiophyceae occurred only in slightly higher amount in grassland soils, except of Schorfheide-Chorin, they clearly predominated in forest soils of Schwäbische Alb and Hainich-Dün Exploratory (Table 5). The recovered OTUs like *Apatococcus*, *Chloroidium*, *Coccomyxa*, *Desmococcus*, *Dictyochloropsis*, *Elliptochloris* and *Trebouxia* are well known aerophytic algae on tree bark and/or lichen symbionts (Ettl and Gärtner, 1995; Freystein et al., 2008; Lüttge and Büdel, 2010; Dal Grande et al., 2014; Hallmann et al., in prep.). *Apatococcus* is among the most widely distributed tree bark algae (Gärtner, 1994) and was exclusively retrieved from the forest soils. Thus it is conceivable that this tree bark alga occurs mainly aerophytic and was washed by rain water from tree bark into the soil. The same may apply to e.g. *Coccomxya*, *Dictyochloropsis*, *Desmococcus* and *Trebouxia*, whereas *Coccomyxa* was found characteristic for acidic soils (Shields and Durrell, 1964). However the OTUs assigned to *Chloroidium* occurred preferably in grassland soil and was also described in soil habitats (Darienko et al., 2010). The *Prototheca*-relative OTUs 31-36 were the most abundant OTUs in forest soils and represented 33.2 % of all clones retrieved from the forest samples. The phylogenetic analysis and Blast search revealed no closer relation to a known green algal genus and represented a distinct group within the Chlorellales (Fig. 2). The phylogenetic analysis revealed a sister-group relationship to the non-photosynthetic genus *Prototheca*, known from various habitats e.g. soil and brackish water and includes parasitic/pathogenic members (Figueroa-Martinez et al., 2015) The next related sequence presented an environmental clone, AOox_H_2012Mar_91 (Acc. No. AB902205), retrieved from a sample of activated sludge treating municipal sewage from Japan (Matsunaga et al., 2014), but with modest blast similarity (Table 4). The exclusive occurrence in forest soils could be explained by low light tolerance or low pH.

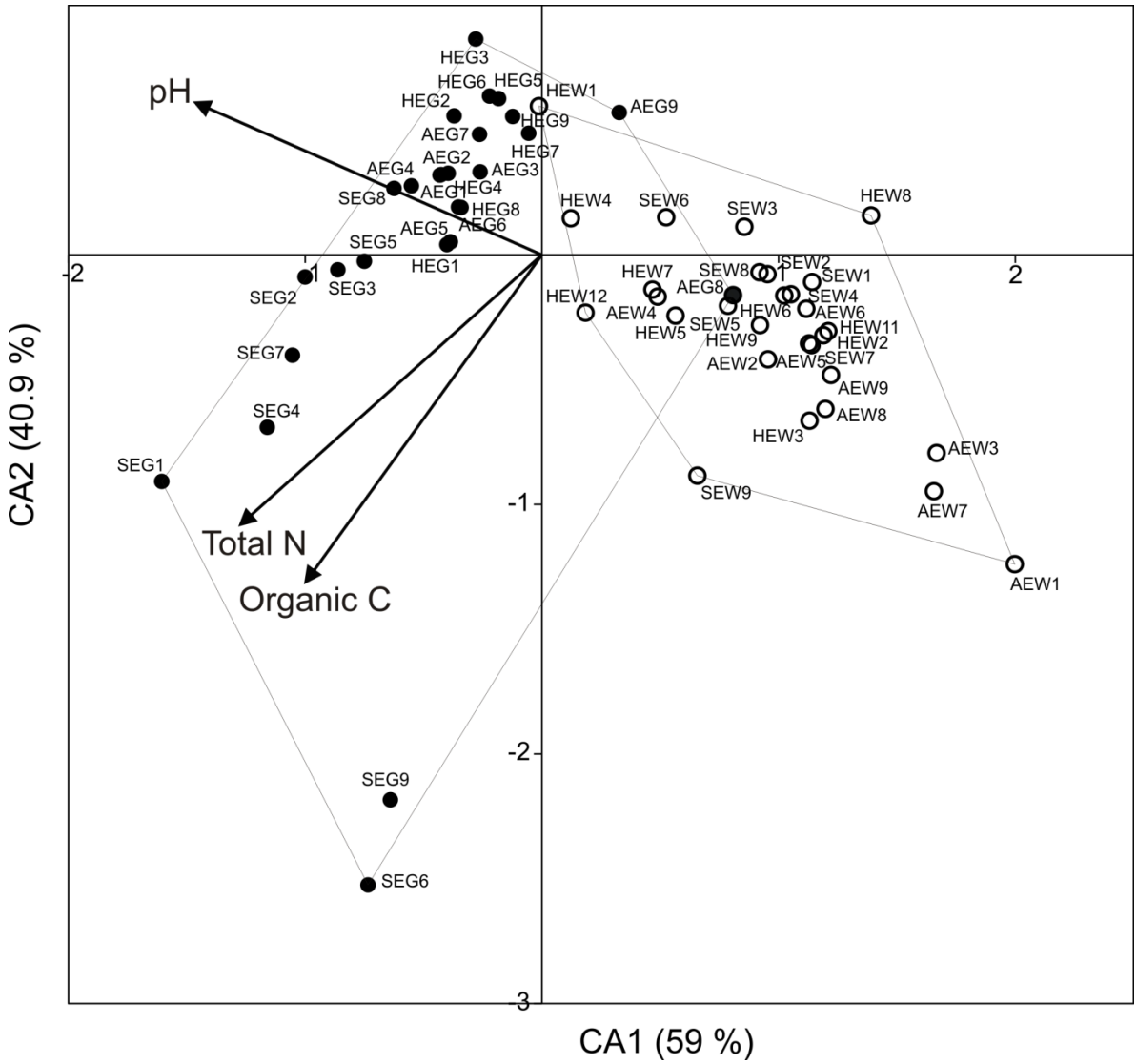

Fig. 6. Canonical Correspondence Analysis (CCA). The ordination diagram showing the grouping of forest (white dots) and grassland (black dots) soil samples in relation to environmental variables (the both canonical axis are significant on the 0.05 level). The analysis was based on a presence/absence matrix of the green algal OTUs.

Management intensities

Three categories of management intensities have been defined for the Biodiversity Exploratories. At the grassland sites were intensively managed meadows (Plot numbers 1-3), managed mown pastures with cattle, sheep or horses as grazers (Plot numbers 4-6) and extensive treated pastures with grazers (Plot numbers 7-9; Table 1 and 2). The managed grassland sites were treated with N fertilization, with a maximum of 140 kg N ha^{-1} yr^{-1} in the Hainich-Dün plots HEG2 and HEG3 (Herold et al., 2014).

The intensively managed grassland sites showed an almost similar OTU richness to the intermediary managed sites with an average of 11.1 OTUs and 11.9 OTUs of each plot,

the extensive treated sites exhibited less diversity with an average of 9.7 OTUs per plot (Fig. 7). In the grassland soils no obvious trend of management intensity of the green alga OTU richness were found. In Schorfheide-Chorin the OTU richness were slightly less at the intermediate managed sites than at the other management intensities, in Hainich-Dün the highest diversity were discovered at the intermediate managed sites, while fewest numbers of OTUs were found at the intensively treated plots. However, at two extensive managed sites in the Schwäbische Alb a very low diversity was obvious: AEG8 exhibited only one OTU, *Chloromonas* OTU 94, with a high number of sequences (35) and AEG9 showed two OTUs with both in a large amount of sequences, *Bracteacoccus* OTU 106 (17 clones) and *Chromochloris* OTU 111 (24 clones), thus OTU 111 occurred exclusively at this site.

The green algal composition revealed no clear gradients to management intensities (CCA; Fig. 6). For each grassland soils a quantitative index of land-use intensity (LUI) was established, quantified as the combined variation of the intensity of fertilization, mowing frequency and intensity of grazing (Blüthgen et al., 2012). The green algal OTUs indicated no significant correlation to LUI (analysis not shown). Allan et al. (2014) demonstrated that multidiversity notably of aboveground organisms decreased with increasing LUI, while belowground groups were less sensitive.

Here the grassland sampling sites with high land-use intensity were treated mainly by mowing up to three times in a year and with N fertilization, while in other studies higher amounts of fertilizers was reported, thus probably no clear trend could be observed (Herold et al., 2014). A former study observed higher cell counts and more genera of soil algae in a newly plowed field compared to an older field (Hunt et al., 1979). Disturbed fields may represent an area open for colonization e.g. by diaspores of airborne algae (Brown et al., 1964; Hunt et al., 1979), this could explain a slightly higher OTU richness in managed grassland sites. Other authors reported algal diversity was found to be greater in the undisturbed sites and disturbance, like tillage and pesticides, had strong effect on the composition and density of algal communities, with cyanobacteria being particularly sensitive to disturbance (Zancan et al., 2006). As well diversity of algal communities in forest and non-forest soils were affected by soil pollutions e.g. lead, coal mines or aerotechnogenic pollutions and less species were observed compared to undisturbed regions (Novakovskaya and Patova, 2007; Patova and Dorokhova, 2008; Temraleeva et al., 2011).

Three categories of forest management intensities were determined, intensively managed spruce or pine age class forest (Plot numbers 1-3), managed beech age class forest (Plot numbers 4-6) and beech selection forest in Hainich-Dün (Plot number HEW7-9) and unmanaged beech forest (Plot number 7-9 and HEW10-12). The OTU richness of green algae indicated at the forest sites of the three regions the same trend. The intermediate managed forest sites exhibited the largest green algal diversities in all three Exploratories (Fig. 7), with an average of 8 OTUs per sampling site of this management intensity, lower diversities were detected in the intensively treated plots with an average of 7.1 OTUs. Interestingly, the lowest diversity was revealed in the unmanaged forest sites with only the half number of OTUs compared to the managed forest sites (an average of 4.1 OTUs).

The unmanaged forest sites were 130 up to 189 years old with well-developed understory vegetation and low light at the ground. Less algal diversity and density in undisturbed forest soils could be explained by high cover of litter and understory and low light intensity at the ground (Hunt et al., 1979; Lukešová and Hoffmann, 1996; Lukešová, 2001). The intensively managed forest sites were coniferous forest and exhibited little less soil algal diversity than in the managed beech forest sites. Differences in algal composition between coniferous and deciduous forest soils were observed earlier, communities of coniferous forest soils were described as less diversified (Neustupa, 2001). At all forest sites of the three Exploratories OTUs affiliated to Trebouxiophyceae dominated, except at the three pine forest sites in Schorfheide-Chorin (SEW1, SEW2 and SEW3). Interestingly ten out of 14 chlorophycean OTUs found at these sites were exclusively recovered in these pine forest soils, while only one trebouxiophyte OTU out of nine were exclusively found at these sites. In Schorfheide-Chorin sandy soils are commonly associated with pine forests, furthermore these three sites showed the lowest values of OC and TN. Land-use, and soil type may correlate and hence both parameters together affect biodiversity (Fischer et al., 2010). However, higher plants also have a significant influence on the soil algal composition (Bakieva et al., 2012). Effects of management intensities on the green algal diversity in soils remain still unclear and are different to separate from physico-chemical parameters of soil.

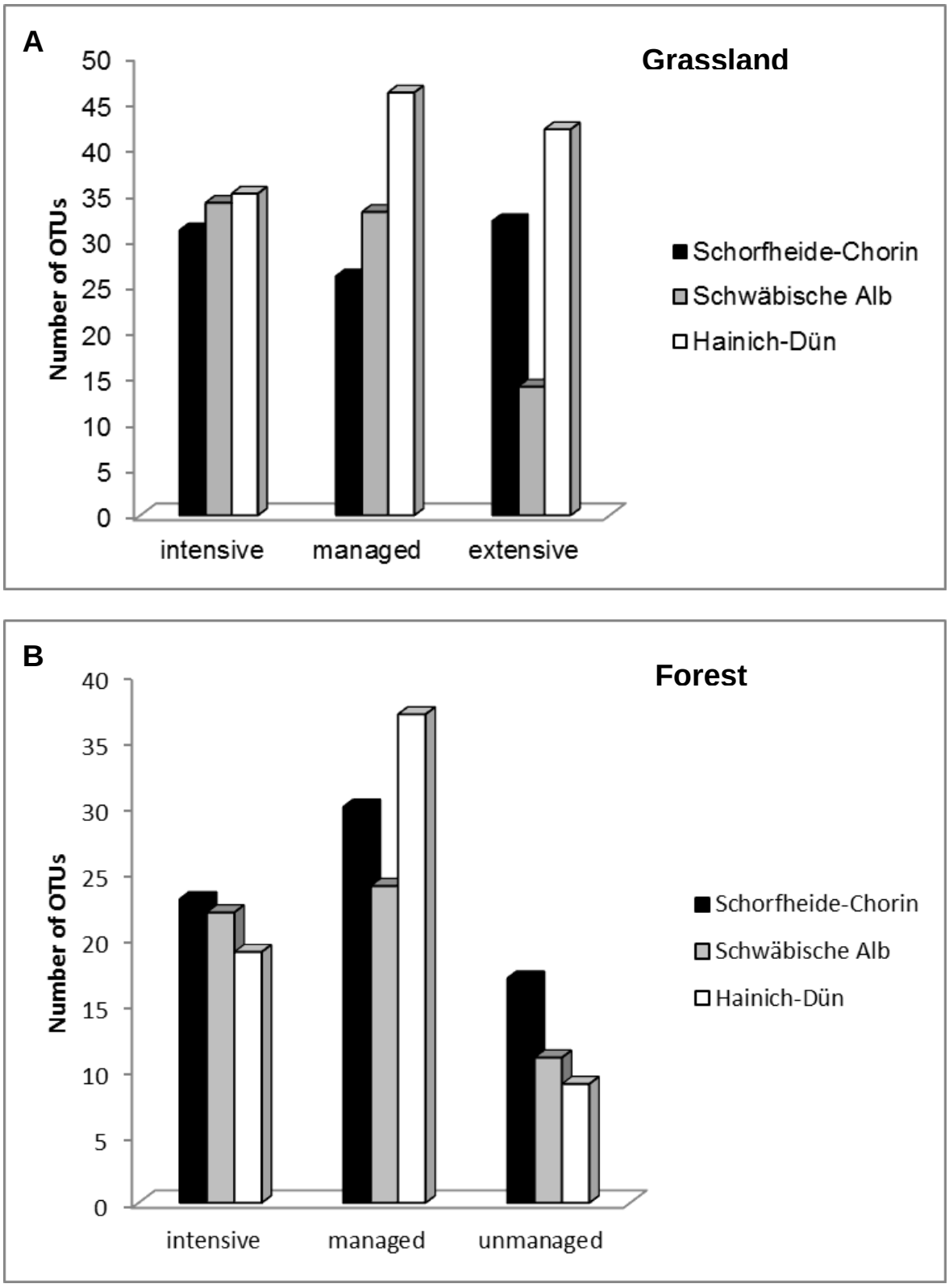

Fig. 7. Distribution of the green algal OTUs in (A) grassland and (B) forest soils of the three German Exploratories. Both land-use types comprised three different management intensities, intensive, managed and extensive/unmanaged.

Sampling effects and seasonal comparison

For a more detailed investigation of the green algal composition in the grassland and forest soils seven plots were selected. From the three grassland plots (HEG7, HEG8 and HEG9) and four forest plots (HEW3, HEW4, HEW5 and HEW10) samples were taken in September 2010 and March 2011, in addition to those from 2008 (Table 6). The topsoil of 0-3 cm was collected; the samples were treated similarly to those 57 samples of the joint effort in spring 2008 (0-10 cm soil depth). Additionally, the March 2011 samples from HEG7 and HEW3 were treated with different methods e.g. using diverse PCR primer combinations and cloning of an enrichment culture obtained from these soil samples. These different efforts were compared to the corresponding samples from spring 2008 with the focus on seasonal comparison, sampling effects, like soil depth and different sampling points at one plot, different treatments of one sampling site to cover the almost complete green algal diversity.

A total of 612 sequences was retrieved from the new sampling effort, altogether 92 OTUs were recovered and 14 OTUs were exclusively found by these approaches (OTU number 146 to 159; Table 4, Table 7). The soil samples taken in September 2010 and March 2011 were treated like the samples from the joint effort and exhibited overall the same trends compared to the samples from 2008. The grassland soils were again found more diverse (eight to 24 OTUs per sample) than the forest soils (six to 14 OTUs per sample) and in the majority of the samples trebouxiophycean OTUs dominated. Additionally same preferences of some OTUs could be observed e.g. *Prototheca*-relative OTUs were preferentially in forest soils, while *Muriella* OTU 42 and *Stichococcus* OTU 54 were recorded mainly from grassland soils (Table 7).

The triplicates within one plot, taken in spring 2008, September 2010 and March 2011, exhibited differences in OTU richness and composition; about 9.7 % to 37.5 % of OTUs were shared at least twice at one plot, whereas sample HEW3 showed the greatest heterogeneity (Table 7). Nevertheless the Detrended Correspondence Analysis (DCA) indicated that the algal communities within one sampling site were more similar and seasonal variations have no significant effect (Fig. 8). Again the algal composition revealed a clear separation of grassland and forest soils, except the forest sample from March 2011 HEW3W. Furthermore the DCA indicated that the green algal composition obtained from the mixed samples from 2008 representing the grassland and forest soils as well.

Table 6. Sample description and numbers of the retrieved green algal OTUs from selected grassland and forest soils of the Hainich-Dün Exploratory.

Plot	Location latitude,	longitude	Land use	Sampling date	Soil depth	PCR primer combination	All sequences	Algal sequences	Non algal sequences (*Dileptus* sp.)	No. of algal OTUs
HEG7S	51°16'25.6" N	10°24'38.5" E	G	September 2010	0-3 cm	20F/CH1750R nested	49	32	17 (1)	21
HEG8S	51°16'16.2" N	10°25'4.6" E	G	September 2010	0-3 cm	20F/CH1750R nested	31	26	5	19
HEG9S	51°13'26.1" N	10°22'51.3" E	G	September 2010	0-3 cm	20F/CH1750R nested	26	17	9 (2)	8
HEW3S	51°16'17.3" N	10°18'37.7" E	F	September 2010	0-3 cm	20F/CH1750R nested	47	42	5 (4)	12
HEW4S	51°22'11.1" N	10°32'1.5" E	F	September 2010	0-3 cm	20F/CH1750R nested	24	20	4	9
HEW5S	51°15'50.9" N	10°14'25.9" E	F	September 2010	0-3 cm	20F/CH1750R nested	37	23	14 (2)	6
HEW10S	51°5'24.6" N	10°27'45.8" E	F	September 2010	0-3 cm	20F/CH1750R nested	25	14	11 (1)	11
HEG7W	51°16'25.6" N	10°24'38.5" E	G	March 2011	0-3 cm	20F/CH1750R nested	36	31	5	19
HEG8W	51°16'16.2" N	10°25'4.6" E	G	March 2011	0-3 cm	20F/CH1750R nested	43	42	1	17
HEG9W	51°13'26.1" N	10°22'51.3" E	G	March 2011	0-3 cm	20F/CH1750R nested	40	38	2	21
HEW3W	51°16'17.3" N	10°18'37.7" E	F	March 2011	0-3 cm	20F/CH1750R nested	36	25	11 (7)	14
HEW5W	51°15'50.9" N	10°14'25.9" E	F	March 2011	0-3 cm	20F/CH1750R nested	45	43	2	7
HEG7MW	51°16'25.6" N	10°24'38.5" E	G	March 2011	0-3 cm	20F/CH1750R nested	39	36	3 (1)	15
HEG7NOW	51°16'25.6" N	10°24'38.5" E	G	March 2011	0-3 cm	20F/CH1750R nested	41	34	7	24
HEG7SWW	51°16'25.6" N	10°24'38.5" E	G	March 2011	0-3 cm	20F/CH1750R nested	40	21	19 (1)	8
HEG7W_B-Horizont	51°16'25.6" N	10°24'38.5" E	G	Spring 2008	B-horizon	20F/CH1750R nested	42	39	3	5
HEG7W_1650Rmod	51°16'25.6" N	10°24'38.5" E	G	March 2011	0-3 cm	20F/CH1650Rmod nested	15	5	10	5
HEG7W_Roh_nested*	51°16'25.6" N	10°24'38.5" E	G	March 2011	0-3 cm	20F/CH1750R nested	33	30	3	13
HEG7W_Roh_18L*	51°16'25.6" N	10°24'38.5" E	G	March 2011	0-3 cm	20F/18L	39	22	17	9
HEW3W_1650Rmod	51°16'17.3" N	10°18'37.7" E	F	March 2011	0-3 cm	20F/CH1650Rmod nested	21	18	3	12
HEW3W_Roh_nested*	51°16'17.3" N	10°18'37.7" E	F	March 2011	0-3 cm	20F/CH1750R nested	33	30	3	4
HEW3W_Roh_18L*	51°16'17.3" N	10°18'37.7" E	F	March 2011	0-3 cm	20F/18L	33	24	9	5

*genomic DNA obtained of six week old liquid enrichment cultures.

Land-use: G: Grassland, F: Forest

Spatial heterogeneity was also investigated at one plot, four samples of the grassland plot HEG7 (NO, SW, the center and the mixed sample, all obtained in March 2011) were analyzed separately (Fig. 8). Altogether 41 OTUs were recovered; the number of the retrieved OTUs varied from eight to 24 OTUs per sample and about 19 OTUs (46.3 %) were shared and discovered in more than two samples. Nevertheless the DCA illustrated similarity in OTU composition at different sampling points within the same plot (Fig. 8).

Seasonal changes in cell counts of soil algae have often been reported (Shields and Durrell, 1964; Metting, 1981; Shimmel and Darley, 1985), whereas in temperate regions a change in soil algal communities is less obvious and more likely due to changing light intensities affected by canopy and leaf litter (Hunt et al., 1979; Johansen et al., 1993; Zancan et al., 2006). Spatial heterogeneity of soil algal communities within one plot is a well-known small and large-scale aspect connected to patchiness of vascular plant cover and also to disturbance caused by e. g. grazing livestock (Grondin and Johansen, 1993; Lukešová, 1993; Lukešová and Hoffmann, 1996; Adler et al., 2001). Whereas these reports were based on morphological investigations, in the here represented study also a PCR or cloning bias has to be taken in account. The sampling in September 2010 resulted, additionally to the samples from 2008, in clone libraries for HEW4S and HEW10S. For these samples nine and eleven sequences were retrieved, whereas only one OTU or no algal sequence was recovered in the samples of the joint effort in 2008.

The sampling site HEG7 was intensively investigated to obtain preferably the best coverage of green algal diversity. Altogether about 304 sequences of this sampling site were analyzed. Additionally to the samples of different seasons and different sampling points, described above, a sample of the B-Horizon obtained from the sampling in 2008 and an enrichment culture established from the sampling in March 2011 were analyzed (Table 6). A total of 60 OTUs were recovered (51 OTUs without the raw cultures). The number of OTUs obtained from the single clone libraries varied from five to 24 OTUs (Table 7), thus the clone library from 2008 exhibited with 21 OTUs a representative sample for this plot. Genomic DNA and green algal PCR-products could also be obtained from soil of the B-Horizon. Five OTUs were recovered, whereas *Chlamydomonas* OTU 73, *Lobosphaera*/*Myrmecia* OTU 20, *Muriella* OTU 42 and OTU 151 were also retrieved in the other clone libraries of HEG7, the Chlorellales relative OTU 46 was exclusively found here (Table 7).

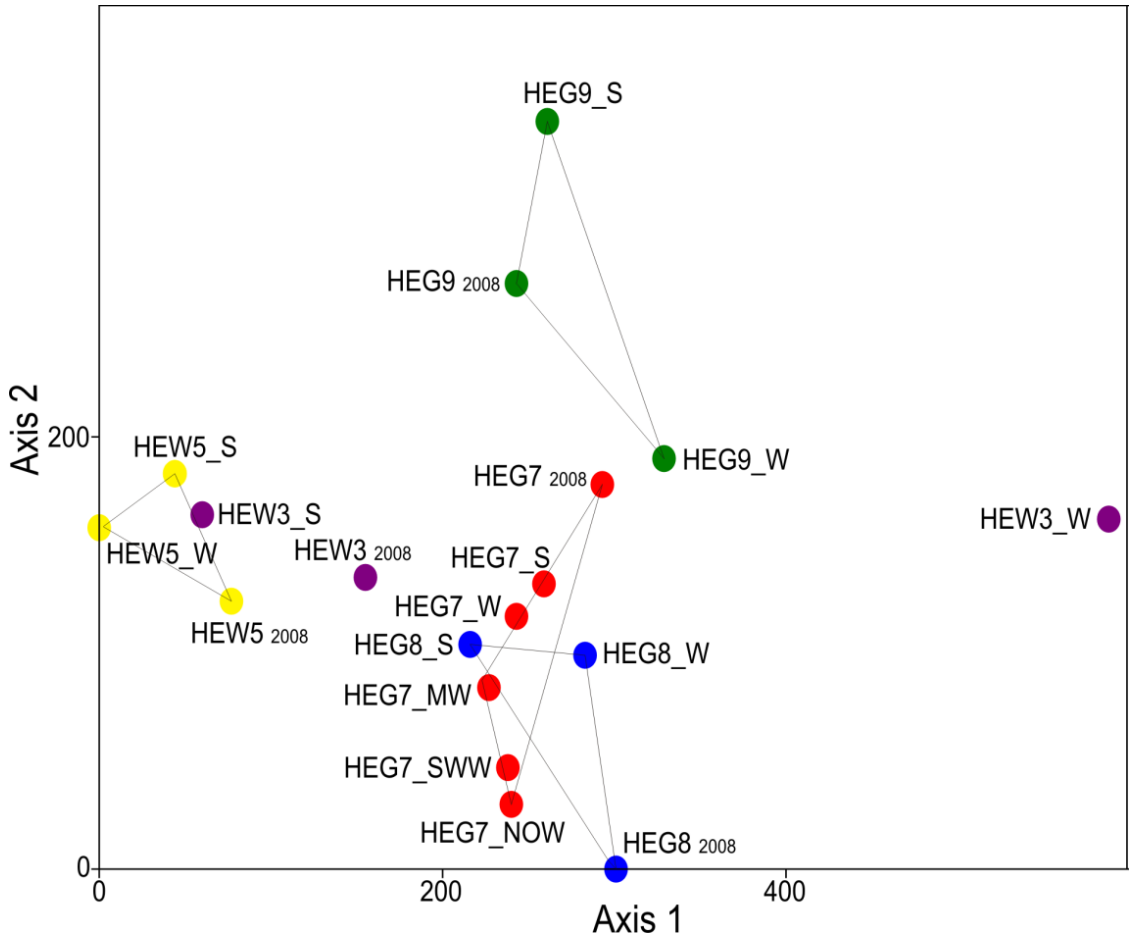

Fig. 8. Detrended Correspondence Analysis (DCA). Comparison of the seasonal green algal OTU-composition in three selected grassland (HEG) and two forest (HEW) sampling sites of the Hainich-Dün Exploratory. The samples were taken in spring 2008 ('2008'), in September 2010 ('S') and March 2011 ('W'). Same colour of dots indicates same sampling site.

Light is a limiting factor for phototrophic soil algae, significant light penetration into soil were reported about 4-5 mm thus the highest soil algae density were in the upper cm and decreases rapidly with depth (Shields and Durrell, 1964; Tester and Morris, 1987; Hoffmann, 1989; Myers and Davis, 2003). Previous studies revealed by culturing also green algal genera like *Chlamydomonas*, *Chlorella* and *Myrmecia* from soil depth up to 18 cm (Willson and Forest, 1957; Fujita and Nakahara, 2006). The presence of algal cells may be feasible up to two meters soil depth caused by water seepage or burrowing activities of animals but same algae were found at surface and in deeper soils (Shields and Durrell, 1964; Hoffmann, 1989). Both approaches, culturing and cloning, may not clarify whether the algal cells were vegetative or resting stages, other adaption strategies were heterotrophy and reducing of catabolic reactions (Metting, 1981; Fujita and Nakahara, 2006). This may also partially explain why the clone libraries obtained from the

enrichment cultures (performed with green algal preferring and eukaryotic PCR primer combinations) differed to those obtained directly from soil samples of plot HEG7 (Table 7). Remarkably the most abundant OTUs recovered from the direct soil samples like *Chloroidium* OTU 15 and *Muriella* OTU 42 were not found in the enrichment cultures. Whereas in the direct soil samples Trebouxiophyceae dominated, the enrichment cultures were more favorable for Chlorophyceae. Generally, the usage of liquid enrichment cultures preferred omnipresent algae (‘weeds’) and restrains the specialist (Hoffmann, 1989; Hoffmann et al., 2007). Furthermore these conditions are beneficial for aquatic species, algae which occur only in low numbers and resting stages, also the presence of Chlorophyceae seems to enhanced (Lukešová and Hoffmann, 1996; Hallmann et al., 2013a, 2015).

Table 7. Distribution of the green algal OTUs in the 18S rRNA gene clone libraries from selected grassland (three) and forest (four) soils of the Hainich-Dün Exploratory. The OTUs belong to the green algal classes of Trebouxiophyceae, Chlorophyceae and Ulvophyceae. Detailed description of clone libraries listed in Table 6.

		HEG7										HEG8			HEG9			HEW3						HEW4		HEW5			
	OTU no.	**HEG7 (2008)**	HEG7S	HEG7W	HEG7MW	HEG7NOW	HEG7SWW	HEG7_B-Horizone	HEG7W_1650Rmod	HEG7W_Roh_nested	HEG7W_Roh_18L	**HEG8 (2008)**	HEG8S	HEG8W	**HEG9 (2008)**	HEG9S	HEG9W	**HEW3 (2008)**	HEW3S	HEW3W	HEW3W_1650Rmod	HEW3W_Roh_nested	HEW3W_Roh_18L	**HEW4 (2008)**	HEW4S	**HEW5 (2008)**	HEW5S	HEW5W	HEW10S
Trebouxiophyceae																													
Apatococcus	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
	146	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
Asterochloris	29	-	2	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Auxenochlorella	39	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
	40	-	1	-	-	1	-	-	-	-	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Chlorella	41	1	-	-	-	-	2	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlorellales relative	43	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	46	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
	49	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlorella mirabilis	60	-	-	1	-	-	-	-	-	10	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chloroidium	15	6	1	3	3	-	1	-	-	-	-	3	-	8	8	-	3	-	-	-	-	-	-	-	-	1	-	-	1
	16	10	1	1	1	-	-	-	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-
	17	12	1	-	-	1	-	-	-	-	-	1	-	3	6	-	7	-	-	-	-	-	-	-	-	-	-	-	-
	18	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coccomyxa	7	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	1	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	1	-	-	5	-	-	1	-	1	-	-	-	-	-	-	-	-
	9	1	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	3	-	3	1	-	-
Desmococcus	50	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	4	5	-	-	-	1	-	-	-	-
Dictyochloropsis	19	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	1	-	-	-	-	-	-	-	-	-	2	-
Dictyochloropsis	149	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Diplosphaera	58	1	1	-	2	-	-	-	-	-	-	-	1	-	3	-	1	-	-	2	1	-	-	-	-	-	-	-	-
Elliptochloris	10	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-	1	-	3	-	-	-	-	1	-	-	-
	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
	12	-	1	-	1	-	-	-	-	-	-	-	-	-	2	1	1	-	-	-	-	-	-	-	-	-	-	-	1
	13	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	2	-	-	-	-
	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-
Fuscochloris	68	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Koliella/Raphidomena	57	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-	-
Leptochlorella	153	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-
Leptosira	66	1	6	-	-	-	-	-	-	-	-	-	-	2	2	1	2	-	-	-	-	-	-	-	-	-	-	-	-
Lobosphaera	20	1	1	1	-	1	-	10	-	-	-	-	1	1	7	-	1	-	2	2	-	-	-	-	3	-	-	-	1
Marvania	45	-	1	2	-	-	1	-	-	-	-	-	1	1	2	-	1	-	-	-	-	-	-	-	-	-	-	-	2
Muriella	42	6	2	3	1	7	2	12	-	-	-	4	1	5	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	151	-	1	-	-	2	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Myrmecia	21	1	-	3	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-
Neocystis	61	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	4	-	-	-	-	-	-	-	1	-	-	-	-
Prasiola	51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
Prototheca	37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Prototheca relative	31	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	3	-	-	-	-	-	-	-	19	12	18	-
	32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	10	-	5	-
	34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7	1	10	-
	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	2	-
Stichococcus	52	1	-	1	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	53	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	3	1	-	-	-	-	-	-	-	-
	54	1	1	3	5	-	-	-	-	-	-	6	1	4	2	-	-	-	-	-	-	-	-	-	-	-	-	4	-
	55	-	1	-	1	1	1	-	-	-	-	1	-	3	-	-	2	1	-	-	-	-	-	-	-	-	-	-	-
	56	-	-	2	5	-	-	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	59	1	-	-	-	-	3	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 7. Continued.

		HEG7										HEG8			HEG9			HEW3						HEW4		HEW5			
	OTU no.	**HEG7 (2008)**	HEG7S	HEG7W	HEG7MW	HEG7NOW	HEG7SWW	HEG7_B-Horizone	HEG7W_1650Rmod	HEG7W_Roh_nested	HEG7W_Roh_18L	**HEG8 (2008)**	HEG8S	HEG8W	**HEG9 (2008)**	HEG9S	HEG9W	**HEW3 (2008)**	HEW3S	HEW3W	HEW3W_1650Rmod	HEW3W_Roh_nested	HEW3W_Roh_18L	**HEW4 (2008)**	HEW4S	**HEW5 (2008)**	HEW5S	HEW5W	HEW10S
Trebouxia	150	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
unident. trebouxiophyte	147	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-
	152	-	5	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Watanabea	148	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xerochlorella	70	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylochloris	69	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	2	-	-	-	1
Chlorophyceae																													
Bracteacoccus	106	2	1	2	-	2	-	-	1	-	-	-	-	1	1	-	3	-	-	-	-	-	-	-	-	-	-	-	-
	107	-	1	1	-	1	-	-	-	-	-	-	-	-	1	-	-	-	5	-	-	1	-	-	-	-	-	-	-
	108	-	-	-	-	-	-	-	-	1	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	2	-	-
	109	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	110	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlamydomonas	72	-	-	-	-	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	73	-	-	-	-	1	-	10	-	-	-	1	-	2	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
	78	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	82	-	-	-	-	-	-	-	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	99	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	124	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlorococcum	71	-	-	1	2	1	-	-	1	-	-	-	-	-	-	-	-	-	4	-	-	10	13	-	2	-	4	-	-
	131	-	-	-	-	-	-	-	-	7	9	-	-	-	-	-	-	-	22	-	-	-	-	-	-	-	-	-	-
	156	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
	158	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Chloromonas	88	-	-	-	-	2	-	-	-	2	1	2	-	-	-	-	-	-	-	-	-	8	3	-	-	-	-	-	-
	89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	1	-	-	-	-	-	-	-	-
	95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
	96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
	98	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	119	-	-	-	3	1	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
	134	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
Chlorosarcina	122	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
Coelastrella	114	1	-	1	-	1	-	-	-	-	-	-	-	-	1	2	1	-	-	-	-	-	-	-	-	-	-	-	2
Coelastrum	115	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Deasonia	113	-	1	-	-	2	-	-	-	-	-	-	-	2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Dictyochloris	140	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	3	-	-
Dictyococcus	125	-	-	2	2	-	-	-	-	-	-	1	1	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heterochlorella	83	-	-	1	4	-	10	-	-	1	2	-	-	-	-	-	-	1	1	-	-	-	-	-	-	1	-	-	1
Oogamochlamydinia	75	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	76	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	1	11	3	-	-	-	-	-	-
	154	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	1	-	-	-	-	-	-	-	-
Pseudomuriella	112	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tetracystis	84	-	1	-	-	1	-	-	-	1	-	-	-	-	2	-	1	-	-	-	-	-	-	-	-	-	-	-	-
	85	-	-	-	-	-	-	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	86	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	101	-	-	-	-	-	-	-	-	2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	105	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	118	-	1	-	1	-	1	-	1	-	-	2	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	120	-	-	-	-	-	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-
unident. Chlorophyceae	92	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
	126	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	157	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Uronema	155	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-
Ulvophyceae																													
Chlorochytrium	143	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scotinosphaera	144	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	3	-	-	-	-	-	-	-	-	-	-	-	-
Pseudendocloniopsis	145	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Printzina	159	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Summary of OTUs		21	21	19	15	24	8	5	5	13	8	12	19	17	26	8	21	8	12	14	12	4	5	1	9	8	6	7	11

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Summary

Terrestrial green microalgae are important primary producers in various habitats like soils, stone (epilithic and endolithic), tree bark and artificial hard substrates. In this thesis, the molecular diversity of green algae (Chlorophyta) in terrestrial habitats was analyzed and assessed by mainly a culture-independent approach using molecular phylogenetics. This allowed the unambiguous re-identification of the same species or molecular operational taxonomic units (OTUs) and, therefore, changes in the taxonomic composition of the algal communities could be reliably assessed. The algal communities in soil and aerophytic algae in phototrophic biofilms on building stone, monuments, on tree bark and on man-made substrate from various sampling sites in Germany were investigated and compared. On building stones and stone monument surfaces also cyanobacteria and fungi were abundant and were analyzed as well. The soil and tree bark samples were obtained from defined research plots of the three German Biodiversity Exploratories, i.e. Hainich-Dün, Schorfheide-Chorin and Schwäbische Alb, which comprised different land use types (forest *versus* grassland) of different management intensities each.

From all sampling sites environmental DNA was isolated and 18S rRNA genes were amplified by PCR with a newly developed green algae preferring primer. Followed by cloning and sequencing the analyses resulted in a dataset of more than 3000 green algal partial sequences. Operational taxonomic units (OTUs) were calculated on the basis of partial sequences (including the hypervariable V4 region) followed by phylogenetic identification of representative full length sequences. For some subsets of samples also enrichment cultures were established and analyzed. In one example, the green algae dominated cover of a man-made hard substrate in an urban environment the different diversities obtained by the culture-independent approach and a culture-based method were compared.

All air exposed surfaces, like stone and tree bark, investigated in this study were clearly dominated by members of the green algal class Trebouxiophyceae. Regarding the studied building stones, the composition of microalgal communities including cyanobacteria differed markedly between apparently similar substrata of two wall areas. This is mainly due to differences in the exposure to sunlight (and hence water availability) and the occurrence of gypsum crusts. Thus, the green algae composition on the daylight-exposed walls was dominated by Trebouxiophyceae, whereas OTUs in samples taken from a sampling site under low irradiance comprised also members of the Chlorophyceae.

Interestingly, members of Chlorophyceae were preferably detected after enrichment in liquid crude cultures. The most abundant algae discovered in phototrophic biofilms on air exposed substrata were various OTUs representing the trebouxiophycean genera *Apatococcus*, *Stichococcus*, *Trebouxia,* and *Coccomyxa.*

The green algal diversity in soils was extensively higher than in aerophytic green biofilms. Significant differences between green algal communities in 27 grassland and 30 forest soils were detected. Besides other factors soil pH explains this observation at best. In relation to management intensities the effects on algal communities were indirect, i.e. soils in unmanaged forests had a markedly lower OTU richness than that of managed forest sites. At the latter, presumably a low light intensity caused by high cover of litter and understory was available to the algal communities which may explain the differences. However, this was not evident for the grassland plots. The soil algal communities comprised a high diversity of members from both classes, the Trebouxiophyceae and Chlorophyceae. The most common OTUs in soil with particular dominance in grassland were species/OTUs of *Chloroidium*, *Stichococcus*, *Muriella*, *Tetracystis* and *Bracteacoccus*. The most abundant OTU in the studied forest soils was a so far unidentified *Prototheca*-related trebouxiophycean alga. This presumably heterotrophic alga was exclusively retrieved from forest soils.

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