**Synechococcus** diversity along a trophic gradient in the Osterseen Lake District, Bavaria

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Picocyanobacteria are important primary producers in freshwater; however, there is still a knowledge gap regarding their diversity at the strain level. For this reason, the microbial diversity of four lakes with different trophic states was investigated by sequencing of the 16S rRNA gene using universal primers. The study was performed in selected lakes of the Osterseen Lake District, Germany, from 2012 to 2014 (Lake Schiffhuettensee: eutrophic; Lake Ostersee: meso-oligotrophic; Lake Groebensee: oligotrophic; Lake Lustsee: oligotrophic). It was determined that the bacterial community of each of these lakes was characterized by one or more specific phyla. Within the autotrophic plankton, the picocyanobacterium **Synechococcus** sp. dominated oligotrophic habitats, whereas eukaryotic algae prevailed in eutrophic lakes. The study focused on the occurrence of cyanobacteria, specifically the genus **Synechococcus**. Genetic analysis of the 16S rRNA gene revealed an extended diversity of freshwater **Synechococcus**. The occurrence of the identified operational taxonomic units of **Synechococcus** did not correlate with the trophic state of their habitat, suggesting that the current, underestimated diversity of picocyanobacteria deserves increased consideration in assessments of microbial and freshwater biodiversity.

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**INTRODUCTION**

Cyanobacteria are important contributors to global primary production; for example, in some marine regions, they can contribute up to 50% of carbon fixation (Li et al., 1983; Li, 1994; Liu et al., 1997; Veldhuis et al., 1997). A subgroup of cyanobacteria, the picocyanobacteria, which are defined by their size of less than 2 µm, are represented by the genera **Synechococcus** and **Prochlorococcus** (Chisholm et al., 1988). Their distribution has been well studied in marine systems (Waterbury et al., 1979; Chisholm et al., 1988; Partensky et al., 1999; Scanlan & West, 2002; Choi et al., 2011; Flombaum et al., 2013; Farrant et al., 2016). According to these findings, **Synechococcus** favours mesotrophic conditions (Zwirglmaier et al., 2007, 2008). Its distribution is still not well understood, and its phylogeny is unclear and needs revision (Dvořák et al., 2014b; Komárek, 2016).

In freshwater, the main picocyanobacteria found are **Cyanobium** spp. and **Synechococcus** spp. (Callieri, 2008), which often cannot be distinguished clearly. **Prochlorococcus**, which dominates oligotrophic regions in marine systems, does not occur. This raises the question whether individual species or strains of **Synechococcus** or **Cyanobium** that occupy the oligotrophic niche in freshwater that **Prochlorococcus** inhabits in the oceans can be identified. Phylogenetic diversity and ecological prevalence studies of freshwater **Synechococcus** are rare and limited to a few lakes (Weisse, 1988; Ernst, 1991; Maeda et al., 1992; Ernst et al., 1995; Callieri & Stockner, 2002; Callieri, 2008; Callieri et al., 2012, 2013; Domaizon et al., 2013). With the advent of high-throughput sequencing, the amount of sequence data from autotrophic picoplankton (APP) has increased exponentially over the last few years (Schuster, 2007; Ansrge, 2009; Metzker, 2010). Efforts to shed light on the phylogeny of freshwater **Synechococcus** have used a number of genes such as the 16S rRNA gene (Urbach et al., 1998; Cro sie
et al., 2003a, b; Ernst et al., 2003; Fuller et al., 2003; Choi & Noh, 2006, 2009; Ivanikova et al., 2007; Sánchez-Baracaldo et al., 2008; Jasser et al., 2011), the ITS-1 region separating the 16S rDNA and 23S rDNA (Ernst et al., 2003; Becker et al., 2004; Choi & Noh, 2006, 2009) and the cpc operons (Crosbie et al., 2003a, b; Ivanikova et al., 2007; Jasser et al., 2011).

In Southern Germany, intensive studies focusing on picocyanobacteria have been conducted only in Lake Constance. Weisse (1988) investigated the vertical distribution and growth of APP. Ernst (1991) and Ernst et al. (1995) analysed pigment composition and genetic diversity of isolated strains. The abundances of APP in the pelagic zone and of Synechococcus-like organisms that were obtained from the phytothentos were studied by Becker et al. (2002, 2004). Additionally, Becker et al. (2007, 2012) investigated the seasonality and annual fluctuations of picocyanobacteria from pelagic and littoral habitats.

The Osterseen Lake District (OLD), Upper Bavaria, Germany, consists of 19 small, interconnected lakes which exhibit a gradient in trophic states, from eutrophic in the south to oligotrophic in the north. Therefore, this lake system is unique for comparative, limnological studies (Melzer, 1999; Raeder et al., 2010; Zwirglmaier et al., 2015). The first comprehensive identification and quantification of microbial diversity in these lakes was carried out in 2012 and suggested that Synechococcus could be the dominant organism within the cyanobacterial population (Zwirglmaier et al., 2015). However, the genetic diversity of picocyanobacteria, as well as the usefulness of their occurrence as a bioindicator of trophic conditions in freshwater, remains largely unknown.

The aim of this study was to determine the diversity of Synechococcus in the OLD. The strains found here were compared to those found in other freshwater lakes to identify new, unique, system-dependent examples. Secondly, we wanted to determine whether the presence of individual clades correlates with the trophic state of the studied lake, as has been found with marine Synechococcus clades (Zwirglmaier et al., 2007, 2008). Lastly, we investigated spatial differences in the composition of the cyanobacterial population along the trophic gradient, as well as seasonal and inter-annual differences. These objectives were realized by analysing the 16S rRNA gene of the microbial community from composite water samples from four lakes of the OLD, which differ in trophic state. Samples were taken twice a year in 2012, 2013 and 2014.

METHODS

Sampling. The four study sites in the OLD of Southern Germany, ca. 50 km south of Munich, are in the Bavarian pre-alpine region. The Lake District consists of 19 interconnected lakes with an anthropogenic trophic gradient from eutrophic in the south to oligotrophic in the north. Four lakes with different trophic states were selected for this study: the eutrophic Lake Schiffauhtensee [total phosphorus (TP): 49 µg l⁻¹], meso-oligotrophic Lake Ostersee (TP: 16 µg l⁻¹), oligotrophic Lake Groebensee (TP: 6 µg l⁻¹) and oligotrophic Lake Lustsee (TP: 6 µg l⁻¹) (Zwirglmaier et al., 2015). For further details about these lakes and the OLD, see Zwirglmaier et al. (2015) and Table S1 (available in the online Supplementary Material).

Sampling took place in August and December 2012, May and September 2013 and March and September 2014. A composite water sample (total volume 1.0 l) from each lake, comprising the entire water column in 1.0 m increments from the lake surface down to just above the sediment, was taken for molecular analysis to provide a cross-section of the microbial community of each studied lake. The samples were kept dark and cool without prefiltration until subsequent processing on the same day. Samples were filtered through cellulose nitrate filters (pore size 0.2 µm, diameter: 47 mm; Whatman/GE Healthcare, United Kingdom) in the laboratory. The filters were stored at −20°C until DNA extraction.

DNA analysis. DNA was extracted from the filters using a phenol/chloroform-based method as described previously (Zwirglmaier et al., 2015) and stored at −20°C until further use.

Partial 16S rRNA genes were amplified with universal 16S rRNA primers and sequenced either by 454 unidirectional pyrosequencing (samples from 2012) as described in Zwirglmaier et al. (2015) or by Illumina MiSeq bidirectional 2×300 paired-end sequencing (samples from 2013 and 2014). The preparation of the samples for Illumina sequencing was done according to the manufacturer’s recommendations (Illumina 16S Metagenomic Sequencing Library Preparation, Illumina, 2013). PCR primers used for the first PCR step were S-D-Bact-0341-b-S-17 (5’-TCGTCGCGCCGCTGAGATGTGATAAGAGA-GCAGCCTACGCGGGCAG-3’) and S-D-Bact-0785-a-A-21 (5’-GTCCTGCTGCGCCGAGGCATTAGCTATAACAGGACTACHVGGGTATCTAAATCCT3’) (Illumina overhang adapter in boldface), which cover the 16S rRNA variable regions V3–V4 (Klindworth et al., 2012). Primers were chosen based on the recommendations of Klindworth et al. (2012), who did extensive in silico studies on primer coverage and suitable primers for sequencing with different amplicon lengths and sequencing platforms. PCR conditions were 95°C for 3 min, 25 cycles of 95°C, 30 s and 55°C, 30 s and 72°C, 30 s, and final extension for 5 min at 72°C using Accelrys DNA Polymerase (Bioline, United Kingdom). The correct size of the PCR products was confirmed by separation with 1% agarose gel electrophoresis. PCR products were purified using AMPure XP beads (Beckman Coulter, USA) according to the manufacturer’s protocol. For the index PCR, the purified products were amplified using the Illumina index primers and the same protocol as for the amplicon PCR, but with only eight cycles. Dual indices and Illumina sequencing adapters using the Nextera XT index kit were attached during this step. Confirmation of the correct size was conducted by comparison of PCR products with MassRuler DNA ladder Mix (Thermo Fisher Scientific, Germany) after electrophoresis on 1% agarose gels. The products were again purified with AMPure XP beads according to the manufacturer’s protocol. Samples were quantified, pooled and then sequenced.

The sequences of 454 Pyrosequencing from 2012 were analysed as described in Zwirglmaier et al. (2015). MiSeq data from 2013 and 2014 were analysed with USEARCH v7.0 and associated python scripts (Edgar, 2010). Quality control of the sequences took place within USEARCH in the following way: first, the forward and reverse reads of each sequence were paired. The reads with low quality and those shorter than 400 bp were removed. The values of the filter parameters were fastq_trunclen=400 and fastq_maxee=0.5. The detection and elimination of chimeras were done using the UCHIME algorithm within USEARCH v7.0 (Edgar et al., 2011). After trimming and checking the quality of the sequences, a total of 732 939 sequences are analysed (Table 1). Sequences, which were determined as 16S rRNA-DNA from chloroplasts, were omitted for the analysis unless specified otherwise.
The clustering levels of the operational taxonomic units (OTUs) were at 97% (for species level) and 99% (for strain level) sequence identity. OTUs were classified by SINA online (Pruesse et al., 2012). Synechococcus OTUs with more than 5% of the total Synechococcus reads were defined as the most abundant OTUs. The phylogenetic tree (Fig. 4) was calculated with ARB (Ludwig et al., 2004) and plotted with FigTree v1.4.2 (Rambaut, University of Edinburgh, UK). Alignments were done in ARB using the SINA plug-in and optimized manually. The tree was constructed by maximum likelihood within ARB, using the sequences from Sánchez-Baracaldo et al. (2008) plus the OTUs found in this study with Illumina sequencing. Only partial sequences of 400 bp covered by the Illumina reads were used for tree construction. Bootstrapping was done with the parsimony interactive tool within ARB. Sequences have been submitted to NCBI (PRJNA318437, PRJNA282452 and PRJNA295806).

PAST (Hammer et al., 2001) was used for statistical analysis. The Shannon index and rarefaction curves were calculated to determine species diversity and richness and evaluate coverage.

**RESULTS**

**Comparison of the relative abundance of major bacterial phyla**

In each of the four lakes, the composition of the bacterioplankton changed only marginally from year to year (Fig. 1).

In all lakes and at every sampling date, mostly β-Proteobacteria dominated, but in some cases, α-Proteobacteria were nearly co-dominant. In addition, Verrucomicrobia occurred in all lakes but their abundance tended to increase with decreasing TP. Regardless, the microbial community of the selected lakes clearly differed, with each lake characterized by characteristic main phyla: Bacteroidetes dominated Lake Schiffhuettensee, and Planctomycetes and Chloroflexi were characteristic phyla for Lake Ostersee, with Planctomycetes contributing up to 25% of all sequence reads (in 2012) in this lake. The composition of the bacterioplankton communities in (the directly adjacent) Lakes Groebensee and Lustsee was similar, although Lake Lustsee had a higher abundance of cyanobacteria (in fact, the highest abundance of all four studied lakes), with up to 16% of sequence reads.

The rarefaction curves (Fig. S1) confirm an almost exhaustive sequencing of the diversity in Lake Lustsee in spring 2014, Lake Schiffhuettensee in autumn 2014 and Lake Ostersee in autumn 2013, whereas the coverage of the diversity in the other samples was considerably lower.

The Shannon index from 1948 is a quantitative measure that reflects the average diversity of, for example, a

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sequencing platform</th>
<th>Bacterial sequences</th>
<th>Synechococcus sequences</th>
<th>Chloroplast sequences (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Schiffhuettensee summer 2012</td>
<td>454</td>
<td>58</td>
<td>0</td>
<td>10 (17)</td>
</tr>
<tr>
<td>Lake Schiffhuettensee winter 2012</td>
<td>454</td>
<td>144</td>
<td>0</td>
<td>71 (49)</td>
</tr>
<tr>
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<td>454</td>
<td>1 321</td>
<td>8</td>
<td>44 (3)</td>
</tr>
<tr>
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<td>454</td>
<td>5 659</td>
<td>20</td>
<td>977 (17)</td>
</tr>
<tr>
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<td>454</td>
<td>2 224</td>
<td>33</td>
<td>325 (15)</td>
</tr>
<tr>
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<td>454</td>
<td>14 903</td>
<td>347</td>
<td>4 143 (28)</td>
</tr>
<tr>
<td>Lake Ostersee spring 2013</td>
<td>Illumina</td>
<td>40 553</td>
<td>2 816</td>
<td>8 807 (22)</td>
</tr>
<tr>
<td>Lake Ostersee autumn 2013</td>
<td>Illumina</td>
<td>120 061</td>
<td>7 415</td>
<td>6 671 (6)</td>
</tr>
<tr>
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<td>5</td>
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<td>13</td>
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<tr>
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<td>17 182</td>
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</tr>
<tr>
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<td>29 294</td>
<td>17 484 (9)</td>
</tr>
<tr>
<td>Lake Lustsee autumn 2014</td>
<td>Illumina</td>
<td>10 243</td>
<td>1 238</td>
<td>1 251 (12)</td>
</tr>
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</table>

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taxonomic unit within an ecosystem. The higher its value (up to 4.5), the more diverse the ecosystem (Shannon & Weaver, 1948). The bacterioplankton of Lake Ostersee was the most diverse, expressed by a mean Shannon index of 2.3 in 2014. The diversity of Lakes Groebensee and Lustsee was similar to that of Lake Ostersee, with Shannon index values of 2.2 and 2.1, respectively. The bacterioplankton of the eutrophic Lake Schiffhuettensee was less diverse, characterized by a Shannon index score of 1.6, the lowest among the four lakes.

Diversity within phylum Cyanobacteria

The sequence data showed an increase in the abundance of cyanobacteria from eutrophic to oligotrophic conditions (Fig. 2). Oligotrophic lakes were dominated by...
picocyanobacteria in contrast to the eutrophic lake, which was dominated by eukaryotic algae (based on sequence reads identified as chloroplasts) and contained almost no cyanobacteria (Fig. 2). It should be noted, however, that the absolute number of eukaryotic cells cannot be concluded from the number of sequence reads of chloroplasts, because of multiple chloroplasts and possibly multiple copies of chloroplast genomes within each eukaryotic algal cell.

Nevertheless, the almost complete absence of cyanobacteria in the eutrophic Lake Schiffhuettensee and the higher abundance of chloroplasts in this lake compared to the other three lakes is a clear indication of differences in primary producers between these lakes. The abundance of cyanobacteria decreased in autumn compared to the spring samples in all four lakes, whereas no clear seasonal pattern was found for chloroplasts. Within the cyanophyta, *Synechococcus* was the most abundant genus. The potential toxin producer *Microcystis* was a further genus within the *Cyanobacteria* found in Lake Ostersee, but it only appeared in small numbers and only in spring. Interestingly, the abundance of *Synechococcus* decreased, while *Planktothrix*, another potential toxin producer, increased in Lake Ostersee in autumn. *Planktothrix* was also found in very low abundance in Lake Groebensee, but it did not increase in autumn like in Lake Ostersee. Cyanobacteria in Lake Lustsee consisted almost exclusively of *Synechococcus*.

**Sequence diversity within the genus *Synechococcus***

The analysis of the genus *Synechococcus* was carried out at the 99% sequence identity level to identify strains. Data, including OTU composition of the mesotrophic and oligotrophic lakes, from 2013 and 2014 from the four lakes were compared where available and are shown in Fig. 3. In total, 49 different *Synechococcus* OTUs were found. Five different OTUs represented the genus *Synechococcus* in Lake Schiffhuettensee, 22 OTUs in Lake Ostersee, 21 OTUs in Lake Groebensee and 41 OTUs in Lake Lustsee. None of the OTUs existed only in one lake. Lake Schiffhuettensee had only a very small number of *Synechococcus* sequence reads; therefore, the results of this lake are not presented in Fig. 3. *Synechococcus* was generally more abundant in oligotrophic waters. OTU ‘OLD2’ was very dominant in all lakes, both in spring and in autumn. Especially in spring, ca. 50% of all *Synechococcus* sequence reads were identified as OLD2. Its abundance generally decreased in autumn, except in Lake Lustsee. OLD27 was present in all lakes and became dominant in autumn in Lake Ostersee in 2013. OLD2968 was more dominant in the oligotrophic Lake Lustsee than in the other lakes. Comparison of the distribution of *Synechococcus* OTUs in Lake Ostersee in 2013 and 2014 shows no distinct differences in spring, but a striking dominance of OLD27 in autumn 2013. All of the most abundant OTUs
appeared in the three lakes ranging from mesotrophic to oligotrophic.

**Phylogeny of Synechococcus OTUs found in the OLD**

A phylogenetic tree of freshwater *Synechococcus*, based on Sánchez-Baracaldo et al. (2008), was reconstructed in order to integrate the *Synechococcus* OTUs from our dataset (Fig. 4). The nomenclature of the clades follows Ernst et al. (2003), Crosbie et al. (2003a) and Sánchez-Baracaldo et al. (2008).

The OTUs found in the OLD formed several new subclusters within the clades defined by Sánchez-Baracrado et al. (2008), particularly within clades I and IV (Fig. 4). Sánchez-Baracaldo et al. (2008) divided clade IV into the groups H and B. Based on our data, clade IV group H is very diverse and divided into different clusters. The highly abundant OLD2 (up to 50% of total *Synechococcus* reads; Fig. 3) belongs to group H. OLD2968, OLD3502 and OLD3962 were categorized also as the most abundant OTUs and formed a new cluster within group H, which is supported by a high bootstrap value (75%). Clade IV group B was expanded by newly identified OTUs with a bootstrap value of 40%, including OLD229. Furthermore, clade III was expanded by another cluster (bootstrap value: 75%). None of the OTUs from our dataset fell into clade II.

**DISCUSSION**

The microbial diversity in general and the diversity of *Synechococcus* in particular of the OLD were investigated by amplicon sequencing of the 16S rRNA gene. As a core finding of this study, we found a strong linkage of *Synechococcus* abundance with oligotrophic conditions. This is in strong contrast to marine systems, where oligotrophic regions are generally dominated by Prochlorococcus, while *Synechococcus* is more dominant in mesotrophic/eutrophic areas (Zwirglmaier et al., 2007, 2008). Therefore, in freshwater, *Synechococcus* could be used as a bio-indicator for nutrient-limited conditions.

The major bacterial phyla within each lake were remarkably stable within the sampling period and showed only minimal differences between spring and autumn, consistent with other studies in the literature (Lindström, 2001; Crump & Hobbie, 2005; Lindström et al., 2005). However, due to the small number of samples taken in this study, we cannot confirm whether this is a general trend. The differences in bacterial diversity between the four study lakes were more obvious, though they reflected typical bacterial distributions of freshwater as found in other freshwater systems globally (Lindström & Leskinen, 2002; Zwart et al., 2002; Newton et al., 2011). Each lake had one or more characteristic main phyla, an observation previously made by Hiorns et al. (1997) in eastern USA. Unsurprisingly, the trophic state appears to play an important role in determining distribution. Jeppesen et al. (2000) showed a unimodal relationship of Shannon index and TP concentration in the context of phytoplankton diversity. The diversity of bacterial phyla correlated similarly with the amount of phosphorus in this study. The oligotrophic Lakes Groebensee and Lustsee had similar environmental conditions and diversity indices. Lake Ostersee, as a mesotrophic lake, was the most diverse and Lake Schiffhüttensee, with the highest phosphorus...
Fig. 4. Maximum-likelihood phylogenetic tree of 16S rDNA sequences from *Synechococcus* and *Prochlorococcus* in freshwater and marine waters *sensu* Sánchez-Baracaldo et al. (2008). OTU analysis level was 99%. Coloured OTUs are from this study and named as OLD. Orange-coloured OLDS are the most abundant, with a relative abundance of >5% of all *Synechococcus* sequence reads. Bootstrap values are shown above nodes; values lower than 50 are not shown. Branch ends show genus and strain names. Accession numbers are in brackets.

Concentration, had the lowest Shannon diversity value. Two different sequencing approaches, 454 Pyrosequencing and Illumina MiSeq, were used for determining bacterial diversity. Due to the nature of the two sequencing techniques, the number of reads obtained from Illumina was higher than that obtained from 454 (Table 1). Furthermore, different regions of the 16S rRNA gene were covered: *Escherichia coli* positions 341–758 in the Illumina data and positions 785–1392 in the 454 dataset. This corresponds to variable regions V3–V4 and V5–V8, respectively. Regardless of this, the results, expressed as relative abundance of individual bacterial phyla, are very similar. An overestimation of the pyrosequencing results relating to the relative abundance of species is possible, but this study shows that the results of both techniques are comparable despite different sequenced regions of the 16S rRNA gene. Some species and phyla could probably not be detected by pyrosequencing because of the lower sequencing depth than Illumina.

Our data showed that the abundance of picocyanobacteria rose steadily with trophic gradient from eutrophic to oligotrophic. Conversely, the abundance of sequence reads from euakaryotic algae decreased along this gradient. This finding supports the model of APP biomass growth with decreasing phosphorus concentrations, as described by Stockner & Shortreed (1991) and Stockner et al. (2000). A possible explanation for the dominance of APP is the small size and hence low surface-size-ratio (Lavin & Lourenço, 2005; Greisberger et al., 2008). Due to this ratio, the organisms are able to absorb and transport nutrients inside their cells more effectively than larger competitors. Another advantage of being small is a low energy requirement (Callieri & Stockner, 2000).
The appearance of *Planktothrix* in oligo-mesotrophic Lake Ostersee in autumn, when the water body was still well stratified, supports the current hypothesis that stable environmental conditions, low nutrient concentrations and a pronounced stratification favour the appearance of *Planktothrix* species as described by Steinberg & Hartmann (1988). Studies concerning the ecology of *Planktothrix* species have been conducted for several decades. Findenegg (1973) correlated the occurrence of this cyanobacterium with increasing nutrient concentrations. Steinberg & Hartmann (1988) challenged the concept that *Planktothrix* occurs due to eutrophication events, which was subsequently supported by the findings of Oberhaus *et al.* (2007) and Van den Wyngaert *et al.* (2011). More recent research addresses questions about its distribution, living conditions and possible impacts of climate change. Mesotrophic lakes as preferred habitat of the toxic *Planktothrix rubescens* were confirmed by further studies. Eutrophication events and restorations of lakes to oligotrophic conditions led to a decline in *Planktothrix rubescens* up to its total disappearance (Dokulil & Teubner, 2012; Jacquet *et al.*, 2014; Garneau *et al.*, 2015; Savichtcheva *et al.*, 2015). It is adapted to low light below the euphotic zone, but passive transport to depth by seiches caused by sustained winds can result in a decrease in daily photosynthetic rate to a negative rate if *P. rubescens* is the main primary producer, as in Lake Zurich (Dokulil & Teubner, 2012; Garneau *et al.*, 2013). Subpopulations of non-microcystin producers prefer the sub-optimal, stressful pressure and light conditions during winter mixis when total *P. rubescens* population is at its minimum (Garneau *et al.*, 2015). Furthermore, warmer springs as a consequence of climate change have a positive effect on the abundance of *Planktothrix* (Savichtcheva *et al.*, 2015).

The seven most abundant OTUs of *Synechococcus* found in the OLD belonged primarily to clade IV group H, with one each to group B and clade I. There was no correlation between the trophic state of a particular lake and individual *Synechococcus* OTUs. This study cannot therefore provide evidence for the hypothesis that particular OTUs prefer defined trophic conditions, as has been shown in marine *Synechococcus* (Zwirglmaier *et al.*, 2007, 2008; Tai & Palenik, 2009; Tai *et al.*, 2011; Huang *et al.*, 2012; Mazard *et al.*, 2012; Pittner *et al.*, 2014; Sudek *et al.*, 2015; Sohm *et al.*, 2016). It is possible that the interconnecting nutrient pathways in this freshwater system have an influence on the *Synechococcus* community structure or that the existing clades are euryoecious. Alternatively, the nitrate-to-phosphorus ratio may play a more important role than simply phosphorus concentration, as Vörös *et al.* (1998) argued. Further research is needed to investigate this hypothesis. The development of the *Synechococcus* population needs to be studied in more detail during a vegetation period.

The results of this study showed that the genus *Synechococcus* has to be split into more lineages than described in previous studies (Honda *et al.*, 1999; Robertson *et al.*, 2001; Crosbie *et al.*, 2003a; Ernst *et al.*, 2003; Sánchez-Baracaldo *et al.*, 2008; Dvořák *et al.*, 2014a). Judging from what has been found in marine systems in the last few years (Schmidt *et al.*, 1991; Urbach *et al.*, 1998; Haverkamp *et al.*, 2009; Scanlan *et al.*, 2009; Mazard *et al.*, 2012), the number of described *Synechococcus* clades is likely to increase further with the availability of more sequence data from freshwater habitats in the future. The tree topology obtained from phylogenetic analysis of our 16S rDNA sequences supported the assumption of Honda *et al.* (1999), Robertson *et al.* (2001) and Dvořák *et al.* (2014a) that *Synechococcus* is polyphyletic and, for this reason, not a natural taxon. This circumstance is evident in the appearance of the same clades in different global ecosystems (Sánchez-Baracaldo *et al.*, 2005, 2008), but the relationship is nevertheless very close within and between the clades (Fig. 4). A discrimination between phycoerythrin- and phycocyanin-rich strains was not possible based simply on sequence data. The new strains of this study were not cultured, and therefore we can say nothing about their pigments as investigated in former studies— for example, Maeda *et al.* (1992), Ernst *et al.* (1995) and Haverkamp *et al.* (2009). Measurements and analysis of pigments were not carried out. These could be topics for further studies.

In conclusion, we characterized the microbial community of four lakes in the OLD between 2012 and 2014. Three previously known freshwater clades of *Synechococcus* could be extended. There are probably still undetected and undescribed clades in other freshwater systems. *Synechococcus* was found in all trophic states but was particularly dominant in oligotrophic habitats, whereas eukaryotic algae prevailed in eutrophic conditions. We could not find a correlation between trophic state and *Synechococcus* OTUs or clades.

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