RESEARCH ARTICLE

Spatio-temporal niche partitioning of closely related picocyanobacteria clades and phycocyanin pigment types in Lake Constance (Germany)

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Abstract

We found that the clade-specific abundance dynamics of *Synechococcus* type picocyanobacteria in the pelagic and littoral zone macro-habitats of Lake Constance (Germany) challenge the hypothesis of a regular annual succession of picocyanobacteria genotypes in temperate zone lakes. Methods used in this study were quantitative *Taq* nuclease assays (TNA), denaturing gradient gel electrophoresis (DGGE), a 19-month time series analysis (with two isothermal and two stratified periods) and genotyping of a new littoral phycocyanin (PC)-rich *Synechococcus* strain collection. The recorded differences between the two macro-habitats and between seasons or years, and the observed effect of water column mixis in winter on the inversion of clade-specific dominance ratios in Lake Constance might explain the known inter-annual differences in abundance and dynamics of the autotrophic picoplankton (APP) in lakes. The APP in Lake Constance shows a high genetic diversity with a low overall abundance, similar to the APP in the Baltic Sea, but different from Lake Biwa in Japan or lakes in the UK. Our results indicate that APP bloom events in both macro-habitats of Lake Constance are driven by phycoerythrin-rich *Synechococcus* genotypes of the Subalpine Cluster I. DGGE revealed the presence of a diverse periphyton (biofilm) community of the PC-rich *Synechococcus* pigment type in the littoral zone in early spring, when no such community was detectable in the pelagic habitat. A more sensitive and quantitative approach with TNA, however, revealed an intermittent presence of one PC-rich genotype in the plankton. We discuss the seasonal development of the pelagic and littoral PC-rich community, and while we cannot rule out a strain isolation bias, we found that isolated PC-rich strains from the pelagic habitat have different genotypes when compared to new littoral strains. We also observed littoral substrates colonized by specific PC-rich *Synechococcus* genotypes.

Introduction

Synechococcus type picocyanobacteria have been described as ubiquitous components of the autotrophic picoplankton (APP) in marine and freshwater habitats, contributing significantly to overall primary production in ecosystems of all climatic zones (e.g. Stockner & Antia, 1986). With respect to their phycobiliprotein composition, two pigment types are known that differ in their phycoerythrin (PE) and phycocyanin (PC) content. Light quality, through its effect on growth rate, is an important factor that triggers the occurrence or co-existence of picocyanobacteria with certain accessory pigments in natural ecosystems (Callieri *et al.*, 1996), and models exist on the importance of the underwater light spectrum and turbidity for the spatial distribution of PE- and PC-rich APP cells (Stomp *et al.*, 2007). PC-rich cyanobacteria can grow at high rates in the presence of high concentrations of

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combined nitrogen (Ernst *et al.*, 1992; Callieri *et al.*, 1996), and they can reach very high abundances in eutrophic/hypertrophic lakes and ponds, shallow parts of lakes, and in cyanobacterial mats or biofilms (Callieri *et al.*, 1996; Vörös *et al.*, 1998; Wakabayashi & Ichise, 2004; Mozes *et al.*, 2006).

Over the past 25 years, we have only started to understand the spatial distribution and genetic diversity of PE-rich APP cells in the pelagic plankton of lakes (e.g. Caron *et al.*, 1985; Padisak *et al.*, 1997; Gaedke & Weisse, 1998; Becker *et al.*, 2002; Crosbie *et al.*, 2003b). Despite a usually low abundance in oligotrophic lakes (Weisse, 1988), an increasing number of studies also focus on PC-rich cells in these ecosystems, but reports on this APP pigment type are still scarce. It was found, for example, that PE- and PC-rich APP cells do occur in the same phylogenetic clusters (Crosbie *et al.*, 2003a; Ernst *et al.*, 2003; Chen *et al.*, 2006) and that both pigment type cells coexist in some ecosystems (Haverkamp *et al.*, 2008); however, differences seem to exist with respect to the abundance and distribution of both pigment types in large lakes and between both habitats and seasons (Fahnenstiel & Carrick, 1992; Becker *et al.*, 2007; Ivanikova *et al.*, 2007).

While a number of studies on marine and freshwater picocyanobacteria (despite some methodological shortcomings, e.g. because of cloning, see Cai *et al.*, 2010) have led to some recent new insights into the clade-specific genetic diversity, seasonal variation, geographic distribution and niche partitioning of communities (e.g. Felföldi *et al.*, 2009; Tai & Palenik, 2009; Winder, 2009; Cai *et al.*, 2010; Wu *et al.*, 2010), freshwater APP in large lakes, and in particular in the littoral zone of such ecosystems, is still understudied. This is surprising, because the littoral zone, with its many ecological niches, may be an important and productive habitat for the whole ecosystem and its microbial communities. Furthermore, the genetic diversity, habitat occurrence, horizontal distribution, abundance and seasonal dynamics of PC-rich picocyanobacteria in lakes and in particular interactions between littoral and pelagic ecosystems, are still poorly understood. For example, insights into the spatio-temporal variation or niche partitioning of bacterial communities in these ecosystems are still scarce, and this holds true at all taxonomic levels, such as phylogenetic clades, genotypes or ecotypes (genotypes represented by isolated strains) and pigment types. In this study, therefore, we revisited the *Synechococcus* picoplankton of Lake Constance (Germany), a large and deep lake of glacial origin that was formed at the end of the last glaciation about 10 000 years ago. In a 19-month time series that includes two isothermal (in Lake Constance from December to March) and two stratified periods (April–November), we provide novel insight into the dynamics, distribution and abundance of littoral and pelagic communities of *Synechococcus* genotypes, focusing on abundance patterns of four closely related clades (Sánchez-Baracaldo *et al.*, 2008). We followed the temporal dynamics of the total PC-rich *Synechococcus* community and one particular genotype of the same pigment type, which both belong to the ubiquitous and highly diverse *Cyanobium gracile* cluster (Ernst *et al.*, 2003) or clade 1 (this study).

Small-scale littoral habitats (or niches) sampled in this study are natural and artificial substrates. The littoral zone is defined as the near-shore area with a water depth between 0 and 10 m, that is, equating to about 13% of the total surface area of Lake Constance (Schmieder, 1997). This habitat also includes the surf zone, with various substrates such as macrophytes, large stones or pebbles. Unlike other studies that lack a background of isolated strains, an advantage of this study was the availability of a collection of clonal, but non-axenic, PC-rich *Synechococcus* strains isolated from both the pelagic and littoral habitat.

In this study, we address the following main points. First, using a time series of spatio-temporal clade variation, we have been able to test the hypothesis of a regular annual succession of picocyanobacteria genotypes in temperate zone lakes, such as Lake Constance (Ernst *et al.*, 2000). Data from two isothermal (December–March) and two stratified periods (April–November) have facilitated analysis of changes in spatio-temporal niche partitioning. Second, the deep quantitative and temporal resolution of possible habitat-related distribution patterns of picocyanobacterial clades and the *Synechococcus* PC pigment type (Becker *et al.*, 2007) provides new insights into the interactions and processes between the pelagic and littoral zone, and the APP community development during seasons. Third, we compared the clade-specific abundance and dynamics pattern of various freshwater and brackish European ecosystems that differ in geological age, size and nutrient status.

Materials and methods

Sampling, strain isolation and culture conditions

All PC-rich *Synechococcus* strains used in this study were isolated from samples from the deep (147 m maximum depth) northwest basin ('Überlinger See') of Lake Constance, Germany, as described previously (Ernst, 1991; Ernst *et al.*, 1995). Becker *et al.* (2004) describe the isolation of periphytic isolates from three natural substrates in the littoral zone of the lake.

Periphyton samples from freshly formed biofilms were obtained from unglazed stone tiles that were exposed for

6 weeks at different water depths of the littoral zone (latitude 47.695321° N, longitude 9.192767° E), 8 km away from the routine pelagic sampling site (latitude 47.76933° N, longitude 9.122086° E) above the deepest part of this lake basin ('Überlinger See'). The tiles were mounted on concrete blocks at a height of 10 cm above the benthos. They were left in the lake for 6 weeks, from the beginning of March to mid-April 2000 at depths of 0.5, 3, 5 and 7 m. After recovery from the lake, each tile was rinsed with 1 L of sterile distilled water to remove sediment particles and unattached cells, and then, the biofilm was brushed off and collected as a cell suspension. Small volumes of these suspensions (50 μL) were spread on BG11:N/3 agar plates, that is, with just 33% of the nitrate present in unmodified BG11 (Stanier *et al.*, 1971), and cultured at 21 °C under low light conditions (5–10 μmol m^{-2} s^{-1}) in white light (Ernst *et al.*, 1992). After 12 weeks, these plates were divided into four segments, and all colonies from each segment were scraped off and pooled for denaturing gradient gel electrophoresis (DGGE) analysis.

To identify (with DGGE) and quantify [with quantitative *Taq* nuclease assays (TNA)] genotypes of isolated *Synechococcus* strains *in situ*, water samples from 'Überlinger See' were collected every fortnight (except on 2 January 2001) between October 2000 and October 2001 from two habitats. An integrated pelagic sample (0–8 m depth), hereafter referred to as 'Pelagic 0–8 m', was obtained from a routine coordinate (see above) above the deepest part of the 'Überlinger See' basin, together with surface water ('Pelagic 0 m'). Surface water samples from the littoral zone ('Littoral 0 m') were taken above a water depth of 5 m at the site where the tiles had been placed (see above). Picoplankton cells from water samples were collected on filters as described previously (Becker *et al.*, 2002).

For the quantification of *Synechococcus* clades 1–4 *sensu* Sánchez-Baracaldo *et al.* (2008) in Lake Constance (Germany), water samples from the surface (0 m water depth) of the pelagic and the littoral zones (February 2001–August 2002) that had already been subjected to TNA analysis for the measurement of total *Synechococcus* genome abundance (Becker *et al.*, 2007) were reanalysed. Surface water samples from ponds and lakes in the UK (Blelham Tarn, Cotswold Water Park 123, Esthwaite Water, and Lake Windermere north and south basin) were collected and analysed for clades 1–4 *sensu* Sánchez-Baracaldo *et al.* (2008).

DNA isolation

Three weeks after inoculation in BG11:N/3 medium, *Synechococcus* batch cultures (40 mL) were harvested by centrifugation (7 min, 7000 ***g***), frozen in liquid nitrogen and stored at −20 °C. Genomic DNA from these clonal samples was extracted as described by Postius *et al.* (1996). DNA from filters with pelagic picoplankton was extracted according to Becker *et al.* (2002). For DNA extraction from periphytic biofilms on tiles, 50 μL of the cell suspension from cultured and pooled colonies (see above) was incubated with 350 μL of 5% (w/v) aqueous suspension of Chelex-100 (sodium form, 100–200 mesh; BioRad, München, Germany) and processed as described (Becker *et al.*, 2002).

Restriction fragment length polymorphisms (RFLP) and PCR genotyping

We used two genome-wide genotyping methods. For RFLP analysis, we used the Southern blotting technique as described by Ernst *et al.* (1995). In brief, 5–10 μg genomic DNA was digested with the restriction endonuclease HindIII and separated on agarose gels. After blotting, restriction fragments were probed with the internal BstEII fragment (labelled with a DIG DNA labelling mix) of *psbAI* from *Synechococcus* PCC 7942 (Hirschberg *et al.*, 1987). Enzymes and detection kits were supplied by Roche (Mannheim, Germany).

For PCR-based genotyping, we used 25 μL PCR assays with 10 ng genomic DNA as a template. Other components of the assays were: 1 mM primer STRR1A (Rasmussen & Svenning, 1998), 1.2 mM dNTP, 5.5 mM Mg^{2+}, 2.5 μL of 10× reaction buffer and 1 U of *Taq* polymerase from Qiagen (Hilden, Germany). PCR was conducted using a PTC-100 thermal cycler (MJ Research, Inc., MA) with the following thermal programme: initial denaturation at 95 °C (3 min), then 30 cycles of 94 °C (1 min), annealing at 56 °C (1 min) and polymerization at 65 °C (5 min). The PCR was terminated with a final elongation step at 70 °C (5 min). A volume of 5 μL from each assay was size-fractionated through a 10% acrylamide gel at 150 V for 5 h in 1× TBE running buffer (pH 8, with 88 mM Tris, 88 mM boric acid and 2 mM Na2-EDTA). Commercial λ DNA digested with PstI was used as a size marker. The gel was stained with Sybr® Gold (MoBiTec, Göttingen, Germany) for 30 min and photographed under UV transillumination.

Denaturing gradient gel electrophoresis

DGGE primers, conditions for PCR and polyacrylamide gel electrophoresis, as well as the gel staining method were used as described in Becker *et al.* (2004). We applied primers that target PC-rich genotypes of the *C. gracile* cluster and Subalpine Cluster II from Lake Constance (Ernst *et al.*, 2003; Becker *et al.*, 2004), and clade 1 *sensu* Sánchez-Baracaldo *et al.* (2008). Another set

of primers detected PE-rich genotypes of Subalpine Cluster I in Lake Constance (Ernst *et al.*, 2003). To confirm their identity, DGGE bands were excised and re-amplified as described by Becker *et al.* (2002): after the migration behaviour of the re-amplified fragments had been checked under similar DGGE conditions, they were bi-directionally sequenced (GATC GmbH, Konstanz, Germany).

Quantitative TNA

Synechococcus clades 1–4 were detected and quantified using quantitative TNA in littoral and pelagic water samples as described in detail by Sánchez-Baracaldo *et al.* (2008). For the enumeration of PC-rich *Synechococcus* sp. strain BO 8805 ribosomal operon (ITS-1) molecules in water samples, we used TNA assays as described (Becker *et al.*, 2004).

Calculations and statistical analyses

To compare clade-specific abundances from this study with total *Synechococcus* genome measurements from an earlier study (Becker *et al.*, 2007), the latter values were multiplied by two, because all *Synechococcus* strains from Lake Constance were found to harbour two ribosomal operons per genome (Ernst *et al.*, 2003). This ensured comparability between the previous data and new clade-specific results, which are in small subunit (ssu, i.e. 16S rDNA in the ribosomal operon) molecules per mL water sample. Before pair-wise correlation analysis, bi-weekly total and clade-specific *Synechococcus* abundance data were subjected to scatter plot analysis to identify linear relationships (data not shown). The data were then log-transformed with the formula [log $(x + 1)$] to obtain an approximately normal distribution of values. Pearson's product-moment correlation coefficient (r) and the non-parametric Spearman's correlation coefficient (ρ) were calculated using the program PASW Statistics, version 18. For calculation of $\log_2$-fold changes between clades, which was used to indicate dominance of one clade over another, we first calculated abundance value ratios (based on absolute abundance) between clades and then plotted the $\log_2$ values of these ratios.

Results and discussion

In this study, we present exemplary new insights into the spatio-temporal dynamics of four closely related *Synechococcus* clades in the APP of Lake Constance, Germany. To our knowledge, this is the first report of a clade- and habitat-specific time series analysis of APP abundance changes that includes the pelagic and littoral habitat of a large temperate zone lake of glacial origin. We used quantitative PCR assays (Becker *et al.*, 2000), developed for a study of pelagic lake APP clades in the UK (Sánchez-Baracaldo *et al.*, 2008). Clade 1 represents the ubiquitous *C. gracile* cluster, of which mainly PC-rich strains have been isolated (Crosbie *et al.*, 2003a; Ernst *et al.*, 2003). Because the pelagic habitat of Lake Constance is dominated by PE-rich *Synechococcus* spp. (Weisse, 1988), our new approach was supposed to detect low abundance of PC-rich genotypes only. Our time series indeed revealed that clade 1 accounted for a maximum of just 16% of all *Synechococcus* genotypes in the pelagic water samples and just 17% in the littoral samples. This is in good agreement (note that a single APP cell may contain several genomes or ssu molecules) with an average 5% PC-rich cells counted in pelagic water samples of the lake using epifluorescence microscopy (Weisse, 1988).

Clades 2 and 3 represent new *Synechococcus* genotypes with unknown accessory pigments, and clade 4 contains PE-rich genotypes that are found in lakes in the UK and in Lake Mondsee in Austria (Sánchez-Baracaldo *et al.*, 2008), another deep subalpine lake of glacial origin at a distance of about 250 km from Lake Constance. The fact that the reverse primer and the probe used in the clade 4 assay contain one mismatch each when compared with the known ssu sequences of PE-rich pelagic *Synechococcus* isolates from Lake Constance (Subalpine Cluster I, Ernst *et al.*, 2003) might explain why this assay detected only up to 50% of all *Synechococcus* ssu mL^{-1} (average 11%) in pelagic samples and up to 51% in littoral samples (average 13%). We conclude that because of the oligonucleotide mismatches (note that a mismatched primer or probe does not inhibit every hybridization event), the clade 4 assay either targets only a fraction of the known PE-rich genotypes in Lake Constance (Subalpine Cluster I, Ernst *et al.*, 2003) or that a periodically large fraction of the PE-rich APP in the lake is formed by so far unknown PE-rich genotypes distinct from clade 4 found in the UK study (Sánchez-Baracaldo *et al.*, 2008).

Clade-specific dynamics of *Synechococcus*

Clades 1–4 appeared to contribute more than 100% to the total *Synechococcus* community on two occasions: 169% in the pelagic habitat on 8 May 2001 (Fig. 1a), and 162% in the littoral sample on 24 April 2001 (Fig. 1b). Other studies that have used TNA and expressed results in cells mL^{-1} (Tai & Palenik, 2009) have seen a similar overestimation in many samples, which might be explained by multiple genomes per cell (Becker *et al.*, 2002). Our data set (ssu mL^{-1}) is robust, with the two outliers possibly explained by an unknown methodological underestimation of the (total) *Synechococcus* abundance in the two original samples (Becker *et al.*, 2007).

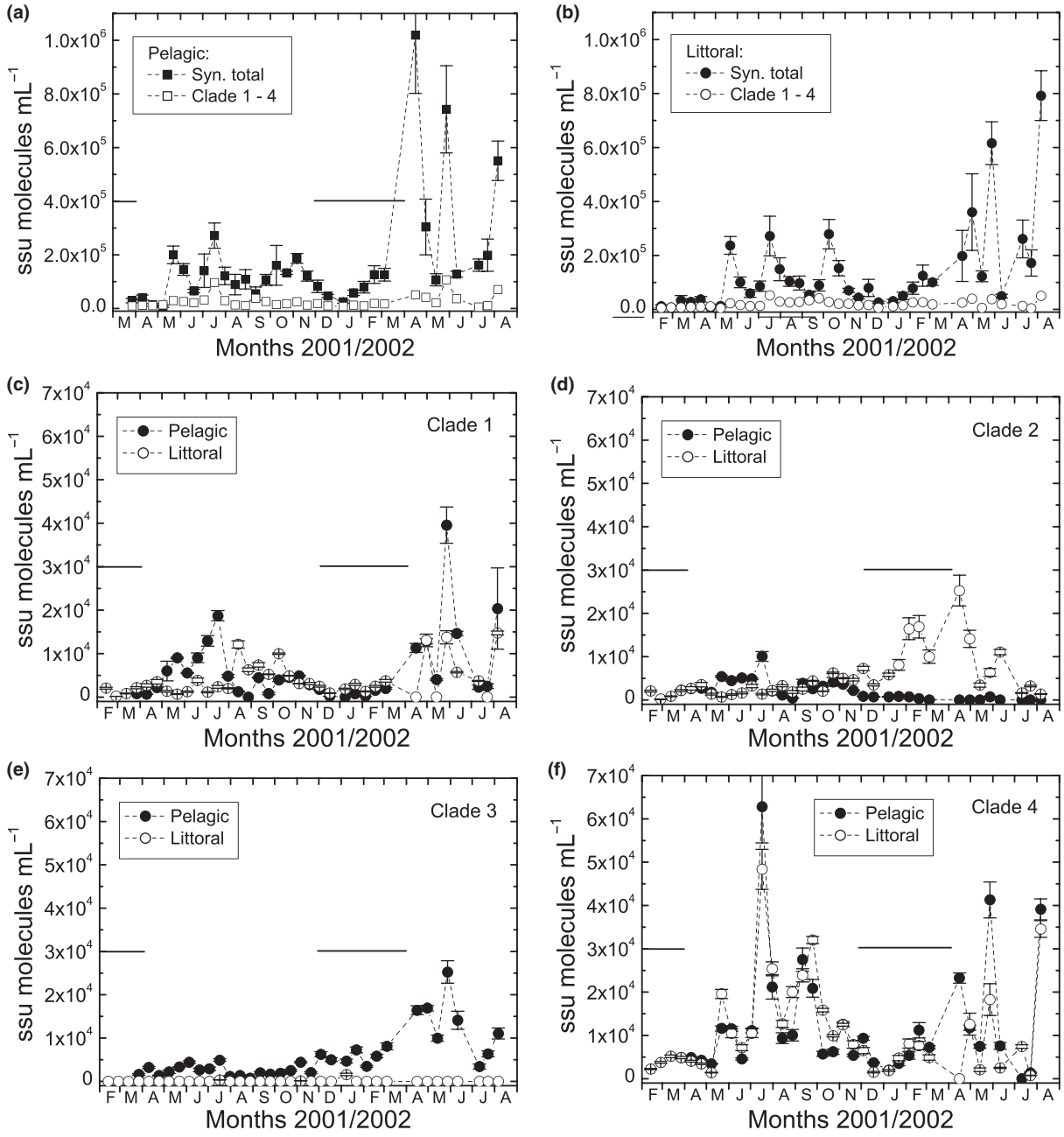

Fig. 1. Total and clade-specific *Synechococcus* abundance in the pelagic and littoral zone of Lake Constance. Determined by TNA, in small subunit (16S rDNA) molecules mL^{-1} water sample from the surface (0 m water depth) of one pelagic and one littoral site (8 km distant). (a) Pelagic site with total and clade 1–4 abundance. (b) Littoral site with total and clade 1–4 abundance. Total *Synechococcus* data in a and b modified from Becker *et al.* (2007). (c–f) Pelagic and littoral site with one clade per panel. Isothermal periods (December–March) are indicated by horizontal lines.

In both the pelagic and littoral zone of Lake Constance, we saw three peaks (blooms) of the total *Synechococcus* community during both stratified periods (Fig. 1a and b), which has been observed before (Gaedke & Weisse, 1998; Becker *et al.*, 2007). While clades 1–4 contributed only a minor fraction during *Synechococcus* blooms in both habitats, all four clades represented a significant portion of the total community during other periods, with an overall contribution of 4–77% in the pelagic habitat (Fig. 1a), and 2–97% in the littoral habitat (Fig. 1b). We conclude that APP bloom events (or strong increases) in Lake Constance are most likely driven by PE-rich genotypes (of

Table 1. Clade 1–4 *Synechococcus* in ponds and lakes in the UK (January 2003–January 2004)

					Number of peaks detected for clade 1–4 (highest abundance in 10^5 ssu mL^{-1})			
Name	Area (ha)	Depth max. (m)	Age (years)	Trophic status	1	2	3	4
Blelham Tarn	10	4	10 000	Mesotrophic	1 (8.8)	n.d.	n.d.	n.d
CWP123*	12	?	17	Eutrophic/mesotrophic	3 (5.0)	n.d.	n.d.	n.d.
Esthwaite Water	195	16	10 000	Eutrophic/mesotrophic	2 (5.5)	3 (1.9)	n.d.	n.d.
Lake Windermere (north basin)	1450	67	10 000	Mesotrophic	2 (5.5)	2 (5.0)	n.d.	n.d.
Lake Windermere (south basin)	1450	67	10 000	Mesotrophic	2 (6.4)	2 (4.3)	n.d.	n.d.

n.d., not detectable in all samples analysed.
*Gravel pond in Cotswold Water Park.

Subalpine Cluster I *sensu* Ernst *et al.*, 2003), of which we might detect only a fraction with the clade 4 assay.

Clade-specific analyses revealed significant temporal abundance differences between the pelagic and littoral habitats on the one hand and between all four clades on the other hand. Clade 1 shows a highly dynamic pattern in both habitats (Fig. 1c). Clades 2 and 3 changed their abundance pattern during the time series: clade 2 was significantly more abundant in the littoral habitat in 2002 (Fig. 1d), while clade 3 gained dominance in the pelagic habitat in the same year (Fig. 1e). Overall, genomes of clade 3 were detectable in only three littoral samples. Clade 4 (PE-rich genotypes) showed a highly dynamic abundance pattern, with only a few significant differences between both macro-habitats (Fig. 1f).

At the pelagic surface of meso- to eutrophic ponds and lakes in the UK, genotypes of clade 1 showed a somewhat dynamic pattern similar to the one in Lake Constance, with up to three distinct peaks and much higher abundance levels (Table 1). Furthermore, in all UK ecosystems, PE-rich genotypes of clade 4 were not detected (Sánchez-Baracaldo *et al.*, 2008). This can be explained by the observed dominance of PC-rich genotypes over PE-rich APP in shallow nutrient-rich ecosystems (Vörös *et al.*, 1998), but it does not explain the lack of PE-rich genotypes in Lake Windermere (deep and mesotrophic). In all freshwater ecosystems sampled in the UK, very defined abundance peaks of only one to three (not all four) clades were detected during periods with otherwise very low (or non-detectable) abundance. However, clades 2, 3 and 4 were much less dynamic than in Lake Constance (Table 1, and see Sánchez-Baracaldo *et al.*, 2008). Hence, the dynamics patterns of clades 2–4 in the UK differed significantly from those observed in Lake Constance (see Fig. 1). This might be explained by the relatively young age (17–30 years) of the man-made ponds studied in the UK. One can hypothesize that in these ecosystems (compared to much older Lake Constance and other UK lakes), only a few APP genotypes might have been introduced and thus been able to gain dominance at times during stratification (Postius & Ernst, 1999), before cyanophages (Deng & Hayes, 2008) or grazers might cause a sharp abundance decline during the annual taxon succession cycle, in agreement with the PEG model (Sommer *et al.*, 1986). We conclude that in small ponds and lakes with a short geological history, the concept of a regular annual succession of APP taxa during the seasons (Ernst *et al.*, 2000) might actually be valid, and data from the UK support this (Table 1, and see Sánchez-Baracaldo *et al.*, 2008).

It seems that 10 000-year-old lakes and ponds of glacial origin in the UK harbour fewer clades with less dynamic abundance patterns than Lake Constance or the brackish Baltic Sea, which is of similar age. All four clades were detected in the pelagic zone of the Baltic Sea during five consecutive days in July 2003 (Sánchez-Baracaldo *et al.*, 2008), and the abundance in that ecosystem was similar to that measured in Lake Constance (this study). Both ecosystems can be regarded similar in terms of physical characteristics (large surface area, deep water bodies that formed about 10 000 years ago at the end of the last glaciation), nutrient status (oligotrophic pelagic area and meso- to eutrophic shore or littoral area, Ojaveer *et al.*, 2010), and with extensive littoral areas providing a variety of substrates. These old ecosystems will have been inoculated many times with APP cells, and they may both provide a richness of ecological niches in which many ecotypes can thrive (Postius & Ernst, 1999), in contrast to (small) eutrophic lakes and ponds in the UK (Sánchez-Baracaldo *et al.*, 2008). Additionally, aquatic ecosystems with a changing nutrient situation over time (from meso-eutrophic to oligotrophic conditions, as in Lake Constance, Kamjunke *et al.*, 2009) or with a gradient of nutrients (oligotrophic pelagic area and meso- to eutrophic shore area, as in the Baltic Sea, Ojaveer *et al.*, 2010) might harbour a wider species spectrum, and also a highly diversified APP, which might explain the highly dynamic spatio-temporal abundance pattern of the clades detected.

Table 2. Pair-wise correlation of bi-weekly total *Synechococcus* and clade-specific abundances (small subunit ribosomal operon copies mL^{-1}) in the pelagic and littoral habitat of Lake Constance

	Pelagic					Littoral				
Variable	Total *Syn*.	Clade 1	Clade 2	Clade 3	Clade 4	Total *Syn*.	Clade 1	Clade 2	Clade 3	Clade 4
Total *Syn*.	–	**0.436***	**−0.417***	**0.548****	0.234	–	0.035	0.049	n.d.	0.210
Clade 1	**0.658****	–	−0.134	**0.388***	0.209	0.288	–	−0.163	n.d.	**0.697****
Clade 2	−0.154	0.149	–	**−0.615****	0.289	0.011	−0.014	–	n.d.	**−0.433***
Clade 3	**0.479***	**0.350***	**−0.557****	–	0.121	n.d.	n.d.	n.d.	–	n.d.
Clade 4	**0.573****	**0.548****	0.165	0.158	–	**0.542****	**0.540****	−0.297	n.d.	–

Pearson's (top) and Spearman's (bottom) correlation coefficients, with significant values in bold.
n.d., not detectable in littoral samples analysed (clade 3).
**Significant at 0.01 levels (two-tailed) with P values < 0.001.
*Significant at 0.05 level (two-tailed) with P values < 0.05.

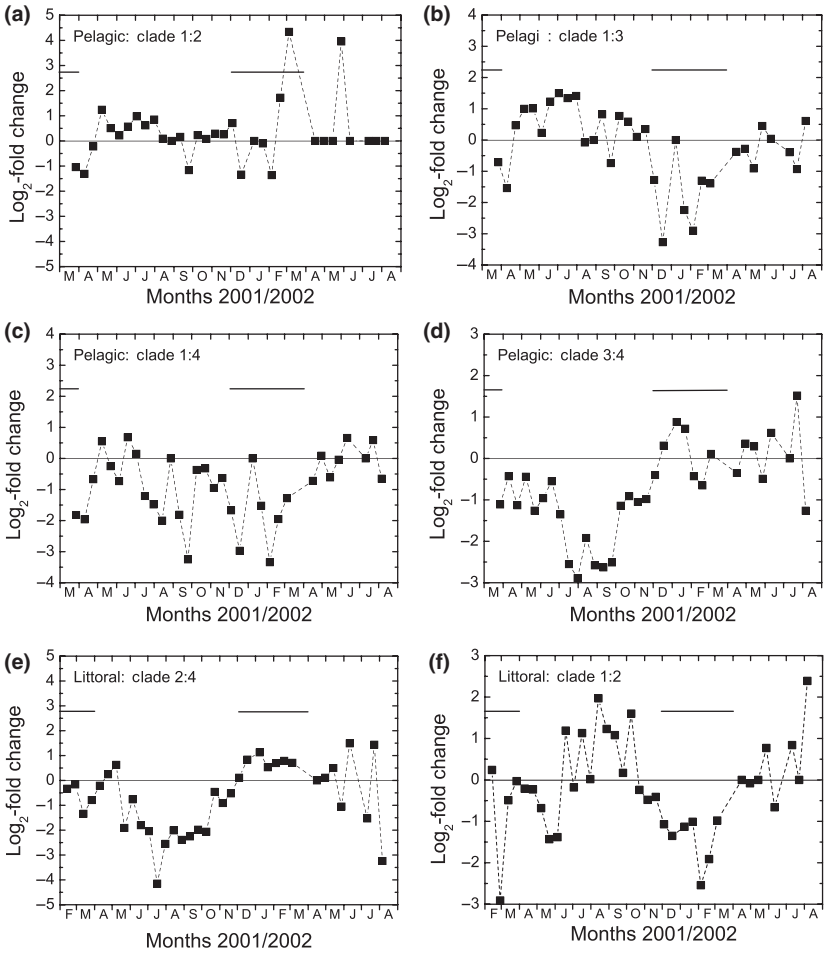

Fig. 2. Log_2-fold change of abundance ratios between *Synechococcus* clades. See panels for clades and habitat (pelagic or littoral plankton). See Materials and methods for data conversion and calculations applied. Isothermal periods (December–March) are indicated by horizontal lines.

There is, however, a clear need for further systematic investigation of the relationships between the geological age and abiotic/biotic factors of a specific freshwater (or brackish) habitat, and the presence, genetic diversity, abundance and dynamics of bacterial communities.

We performed a correlation analysis of clade-specific abundance dynamics, revealing significant differences between the pelagic and littoral habitat of Lake Constance. In the pelagic habitat, an abundance increase in clade 1 is significantly correlated with an increase in clade

3 and both are significantly correlated with a total *Synechococcus* increase (Table 2). These results are reflected in an analysis of the $\log_2$-fold change between clades (Fig. 2). Figure 2a shows dominance of clade 1 over clade 2 during spring/summer stratification, and Fig. 2b shows a dominance of clade 3 over clade 1 during mixis or isothermal conditions (March 2001 and December 2001–March 2002). Clade 4 dominates over clade 1 most of the time (Fig. 2c); note that only Spearman's correlation coefficient showed a significant correlation between an increase in clade 4 and total *Synechococcus* (Table 2). This is proof that the major factor in an APP increase in the pelagic habitat of Lake Constance is indeed the PE-rich genotype community (clade 4).

Deep mixis or isothermal conditions of the water column have the potential to invert dominance ratios between years, for example, between clade 3 and 4 in Lake Constance (Fig. 2d). We explain this with a change in the inoculation rate of the water column, which might be related to cell recruitment rates from sediments. It is also possible that the mixing depth in winter recruits or selects certain genotypes that have an advantage in the following growing season. This would be similar to the selection of gas vesicle genotypes of filamentous cyanobacteria in other deep monomictic alpine lakes, such as Lake Zurich (Walsby, 1994). If winter mixis can have such a striking effect, our observations might explain the large inter-annual differences in pelagic APP abundance in Lake Constance (Gaedke & Weisse, 1998) and would challenge the concept of a regular annual succession of APP genotypes (Ernst *et al.*, 2000).

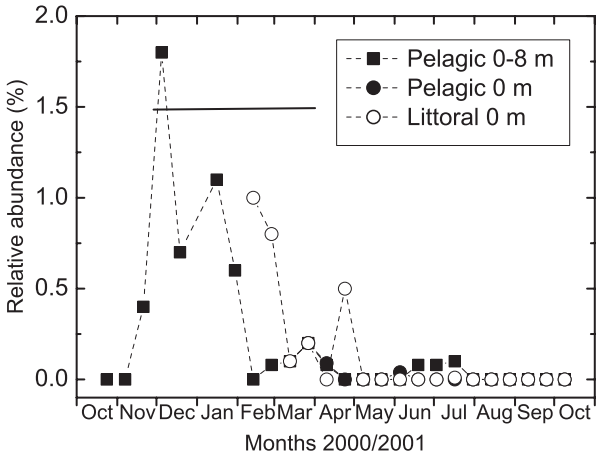

Fig. 3. Relative abundance of PC-rich *Synechococcus* sp. BO 8805 in Lake Constance. October 2000–October 2001, water samples as indicated. Quantification with TNA, for details see Materials and methods section. The mean genome number for each data point ($N = 6$) of strain BO 8805 was divided by the mean genome number of all *Synechococcus* spp. in the same sample (Becker *et al.*, 2007). The isothermal period (December–March) is indicated by a horizontal line.

PC-rich *Synechococcus* of the *C. gracile* cluster (clade 1) in Lake Constance

The importance of PC-rich APP in the littoral zone of Lake Constance (Becker *et al.*, 2004), and the ubiquity and genetic diversity of genotypes of the *C. gracile* cluster (Ernst *et al.*, 2003), prompted a more detailed study of the genetic diversity of new strains and the spatio-temporal niche partitioning of clade 1. This clade shows a highly dynamic pattern in both the pelagic and littoral zones of Lake Constance (Fig. 1c), which is reflected in the dynamics pattern of one genotype from this clade, PC-rich pelagic (isolate) *Synechococcus* sp. BO 8805 (Becker *et al.*, 2004), see below. Clade 1 genotypes are present in both macro-habitats during all seasons (Fig. 1c), with strain BO 8805 predominantly present in both habitats during the isothermal period (December–March, Fig. 3), where it contributes a maximum of 40% to total abundance of clade 1 at the end of February 2001. Interestingly, strain BO 8805 was below the detection limit in all samples after August 2001 (Fig. 3, and data not shown), another observation that challenges the notion of a regular annual succession of APP genotypes in lakes (Ernst *et al.*, 2000).

Correlation analysis revealed differences in the clade-specific abundance dynamics between the pelagic and littoral habitats of Lake Constance (Table 2). In the littoral zone, an increase in clade 4 abundance was significantly correlated with an increase in total *Synechococcus* (only Spearman's correlation coefficient was significant), but clade 1 was also significantly correlated with clade 4. These results are also reflected in the analysis of the $\log_2$-fold change between clades, with clade 4 dominant over clade 2 (Fig. 2e) during peaks of total *Synechococcus* abundance while the lake is stratified (compare with Fig. 1b), and clade 1 vs. clade 4 not showing any oscillation (data not shown). Clade 1 often was dominant over clade 2 during the two periods of water column stratification (Fig. 2f), with the true dominance of clade 1 (values higher than 0) coinciding with the second and third peak of total *Synechococcus* abundance during stratification, and not with the first peak (i.e. the spring bloom: compare with Fig. 1b). We conclude that the major contributors to planktonic APP abundance increases in the littoral zone, particularly during the spring bloom, are PE-rich *Synechococcus* (clade 4), with PC-rich *Synechococcus* (clade 1) making a significant contribution during later periods of high (or increasing) total abundance.

We examined in more detail the genetic diversity and habitat presence of established pelagic and new littoral

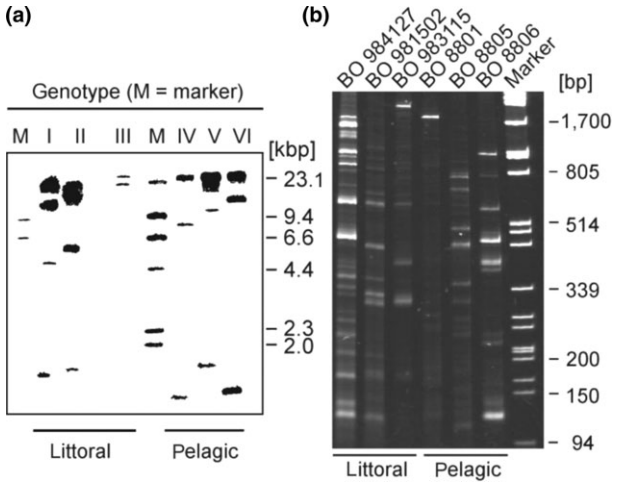

Fig. 4. Genotyping of PC-rich *Synechococcus* spp. from the littoral and pelagic zone of Lake Constance with RFLP and PCR. (a) RFLP, genomic DNA was digested with HindIII and probed with an internal fragment of *psbAI* from *Synechococcus* PCC 7942. I to VI, genotypes; M, DIG-labelled molecular weight marker II (Roche). (b) For PCR, 10 ng genomic DNA of each genotype identified by RFLP (*Synechococcus* spp. strains BO 984127, BO 981502, BO 983115, BO 8801, BO 8805, BO 8806) was amplified with primer STRR1A, 5 μL of the assay was separated on a 10% acrylamide gel. λDNA digested with PstI was used as molecular marker. For details, see Materials and methods section.

PC-rich *Synechococcus* isolates in our strain collection from Lake Constance. Genome-wide RFLP analysis led to the identification of three distinct genotypes when genomic DNAs of ten periphytic (littoral) PC-rich *Synechococcus* strains (Table 3) were digested with HindIII (Fig. 4a): all isolates obtained from biofilms on the macrophyte *Phragmites australis* were of genotype I, three strains isolated from a single stone belonged to genotype III, and strains from the macrophyte *Chara* sp. were of genotype III (one isolate) and genotype II (two isolates). The PC-rich genotypes IV–VI from the pelagic zone, established previously by RFLP analysis using BamHI, EcoRI and SalI (Ernst *et al.*, 1995), were also confirmed in this study by digestion with HindIII (Fig. 4a, Table 3). None of the littoral genotypes were similar to those from pelagic isolates. These results were confirmed independently using a time- and resource-consuming PCR-based genome-wide cyanobacterium genotyping technique developed by Rasmussen & Svenning (1998): Fig. 4b shows six different band patterns, that is, genotypes that correspond to the RFLP results. This PCR method can be applied for quick genotyping of new isolates, because only a few cells from single colonies provide enough source material, circumventing the need for DNA extraction (data not shown). We conclude that genetic diversity is high among *Synechococcus* spp. on natural littoral substrates during thermal stratification in late spring and summer, and we propose an association between certain genotypes and substrates. However, we cannot rule out a possible strain isolation bias (Becker *et al.*, 2004), and our results are very preliminary in terms of the number of isolated strains and time period sampled. Furthermore, we were not able to isolate any PE-rich strains from natural littoral substrates, at a time (June and July of the year 1998) when the picoplankton of Lake Constance was dominated by PE-rich *Synechococcus* spp. (Weisse, 1988; Becker *et al.*, 2002, 2007).

To explore whether the PC-rich *Synechococcus* genotypes in littoral periphyton of Lake Constance during spring (Becker *et al.*, 2004) might provide an inoculum for the littoral or pelagic APP plankton, we studied biofilms developed on unglazed tiles that had been placed 10 cm above the littoral benthos of the lake. First, cultures derived from these biofilms were analysed by DGGE. Also here we cannot rule out a genotype isolation bias with this approach, but within an abundant and diverse biofilm community we observed co-dominance of several genotypes (similar amplicon band patterns) at all four water depths sampled (0.5, 3, 5 and 7 m), and all DGGE gels (data not shown) resulted in a band with the same R_f value as the amplicon derived from strain *Synechococcus* sp. BO 8805: the presence of this specific genotype was confirmed by sequence analysis (GenBank accession AF317073). Second, we then used DGGE to screen water samples from Lake Constance collected during mixis or isothermal conditions. We found a diverse community of known PE-rich *Synechococcus* spp. genotypes in pelagic water samples in February 2001 only, whereas during the same sampling period (October 2000–April 2001) a PC-rich community of known genotypes was not detectable (data not shown). A much more sensitive and quantitative approach using qPCR (TNA), however, revealed the presence of BO 8805 genomes in most of these and other pelagic samples, with peaks during mixis or isothermal conditions and during the clear-water phase in June and July (Fig. 3). Between March and October 2001, there was no significant difference between both pelagic samples (surface and integrated sample), except during the clear-water phase. At the surface of the littoral zone, we observed a significantly higher relative abundance of strain BO 8805 during mixis or isothermal conditions (February–April) than during thermal stratification.

These results demonstrate that during mixis or isothermal conditions (December–March), PC-rich *Synechococcus* prevail in the littoral zone of Lake Constance, and specific genotypes of this pigment group might show a relative abundance peak during this period. Reasons for this may be the presence of favourable nutrient, temperature or light conditions, or ecological niches in a wider

Table 3. Littoral and pelagic PC-rich *Synechococcus* strains and genotypes from Lake Constance

Strain	Sampling date (day/month/year)	Habitat, surface	Genotype*
BO 984124	09/06/1998	Littoral zone, *Phrag. aus.*†	I
BO 984125	09/06/1998	Littoral zone, *Phrag. aus.*†	I
BO 984126	09/06/1998	Littoral zone, *Phrag. aus.*†	I
BO 984127	09/06/1998	Littoral zone, *Phrag. aus.*†	I
BO 983115	07/07/1998	Littoral zone, *Chara* sp.	II
BO 983117	07/07/1998	Littoral zone, *Chara* sp.	II
BO 983120	07/07/1998	Littoral zone, *Chara* sp.	III
BO 981502	09/06/1998	Littoral zone, single stone	III
BO 981503	09/06/1998	Littoral zone, single stone	III
BO 981505	09/06/1998	Littoral zone, single stone	III
BO 8801	1988	Pelagic zone	IV
BO 8805	July 1988	Pelagic zone	V
BO 8806	July 1988	Pelagic zone	VI
BO 9301	15/05/1993	Pelagic zone	IV
BO 9302	27/04/1993	Pelagic zone	IV
BO 9303	27/04/1993	Pelagic zone	IV

*Compare Fig. 4.
†*Phragmites australis.*

sense (Postius & Ernst, 1999), for example, substrates or biofilms (Becker *et al.*, 2004). Furthermore, near-shore lake water may provide better growth conditions for PC-rich APP cells, as was demonstrated in Lake Baikal (Katano *et al.*, 2008). Pelagic PC-rich *Synechococcus* isolates from Lake Constance were shown to respond to N-deprivation by exudation of high-molecular weight polysaccharides, a feature not detectable in PE-rich strains (Postius & Böger, 1998). These pelagic PC-rich isolates (e.g. strain BO 8805) might originate from detached littoral cells and exuded polysaccharides might lead to attachment to littoral substrates and the formation of biofilms. In PE-rich strains isolated from the deep littoral section (7 m water depth) of Lake Constance (Becker *et al.*, 2004), high light stress leads to a significant reduction in the chlorophyll per cell, which was not observed in PC-rich strains from the upper littoral zone of the lake (Strittmatter, 2006). Callieri *et al.* (2005) and Moser *et al.* (2009) demonstrated that pelagic PC-rich *Synechococcus* isolates from Lake Constance (e.g. strain BO 8801, see Table 3 and Fig. 4) are better adapted to high light irradiance than PE-rich strains from the same phylogenetic cluster, which is in agreement with the idea that there is probably a difference between the two pigment types with respect to niche adaptation in the same habitat. More physiological studies are needed in this field, but PC-rich *Synechococcus* in the upper section of the littoral zone of Lake Constance (e.g. certain genotypes on macrophytes in the surf zone) may be adapted to stress conditions such as nutrient depletion and/or high light. In marine APP, light seems to be an important selecting factor in the water column (Six *et al.*, 2007). This may also hold true in the littoral areas of lakes, where high light conditions may lead to the dominance of certain PC-rich *Synechococcus* genotypes (e.g. in biofilms on macrophytes in the surf zone), and where the presence of certain nutrient (Katano *et al.*, 2008) or physical conditions such as temperature regimes (Sommer, 1985; Schweizer, 1993; Gaedke & Weisse, 1998), and substrates (Becker *et al.*, 2004) might provide suitable conditions in the littoral habitat as well.

Similar to the PE-rich genotype BO 8807 (Becker *et al.*, 2007), the model PC-rich genotype BO 8805 in this study shows higher relative abundance during mixis or isothermal conditions in winter (December–March) and when the total *Synechococcus* spp. abundance is low, for example, during the clear-water phase in June and July (Fig. 3). Hence, there are genotypes of both pigment types that may have an advantage over others during isothermal conditions. In an earlier study, we had demonstrated that during summer, there is indeed a detectable and highly diverse community of PC-rich *Synechococcus* spp. present in the pelagic area of Lake Constance (Becker *et al.*, 2004). It is not known whether these PC-rich pelagic *Synechococcus* are detached genotypes from the spring biofilms in the littoral zone or whether certain PC-rich APP genotypes persist in the pelagic zone during thermal stratification. The clade 1 results (Fig. 1c) support the latter assumption.

Concluding remarks

Recent new findings such as endemic bacterial populations (Salcher *et al.*, 2011), rapid local speciation (Pereyra *et al.*, 2009), ecosystem-dependent adaptive radiation (Ernst *et al.*, 2003) challenge established observations in microbial ecology. Other examples include significant differences in the presence of microbial genotypes and clades within and between ecosystems and habitats (Becker *et al.*, 2007; Choi & Noh, 2009; Caravati *et al.*, 2010), the distribution pattern of marine *Synechococcus* clusters in Chesapeake Bay (Chen *et al.*, 2006; Cai *et al.*, 2010), and differences in the abundance of (novel) picocyanobacteria clusters between the near-shore and pelagic area of Lake Superior (Ivanikova *et al.*, 2007). The results presented in this study are consistent with these observations, and we provide exemplary new findings with respect to the spatio-temporal PC-rich *Synechococcus* community in Lake Constance.

Our clade-specific results, in particular the observed inversion of dominance ratios with water column mixis (December–March), might explain the observed interannual pattern of abundance differences in the total

Synechococcus community in the pelagic habitat of Lake Constance (Gaedke & Weisse, 1998; Becker *et al.*, 2002) and other deep monomictic lakes. Our results also demonstrate that such mechanisms might affect the pelagic or littoral habitat only, certain clades (absence of clade 3 in littoral samples, see Fig. 1e), or either the PE- and PC-rich APP pigment types, which would lead to significant abundance differences between them, in particular during the onset of thermal stratification and the following spring bloom and later periods of high or increasing abundance. The question about whether there is a regular annual succession in the APP of temperate zone lakes (Ernst *et al.*, 2000) remains unanswered, but our results appear to challenge this hypothesis, at least when lakes and ponds in the UK are compared with deep monomictic lakes, such as Lake Constance.

Clade 4 harbours widespread genotypes present in Lake Biwa in Japan (4 million years old), Lake Constance (Germany), Lake Mondsee (Austria), and the Baltic Sea (all about 10 000 years old), and lake 124 in Cotswold Water Park, UK (18 years old) (Sánchez-Baracaldo *et al.*, 2008). More studies are needed to understand how the diversity and history of this clade (and others) correlate to distribution and abundance patterns observed in this and other studies (e.g. Wakabayashi & Ichise, 2004; Sánchez-Baracaldo *et al.*, 2008). It has been proposed that microbial diversity is particularly high in lakes that emerged from landscapes after deglaciation (Caravati *et al.*, 2010). We have seen this in Lake Constance (and the brackish Baltic Sea). However, at least periodically, and when compared to other lake ecosystems (for example in the UK), the lowest APP abundance seems to occur in these two large post-glacial ecosystems of intermediate age.

Recent phylogenomic and relaxed molecular clock analyses have suggested that the SynPro clade (cyanobacterial clade with unicellular *Synechococcus*, *Prochlorococcus*, *Cyanobium*) emerged in the Neoproterozoic, about 600 million years ago (Sanchez-Baracaldo *et al.*, 2005; Blank & Sánchez-Baracaldo, 2010). With this in mind, it is still unclear how major ecosystem changes (e.g. glaciations) have influenced microbial populations, and in particular freshwater picocyanobacteria communities. Furthermore, recent molecular ecology studies (e.g. Becker *et al.*, 2002, 2004, 2007; Crosbie *et al.*, 2003b; Sánchez-Baracaldo *et al.*, 2008) have revealed complex and diverse freshwater APP communities in need of further studies, also with respect to scenarios and models of future changes, for example, because of climate change, which might have major effects on important primary producers such as APP. Quantitative methods described in this paper, and new technologies such as single DNA molecule counting with next generation sequencing techniques (e.g. Quince *et al.*, 2009), will be cutting-edge tools in such challenging studies.

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