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Seasonal dynamics of heterotrophic and plastidic protists in the water column of Lake Biwa, Japan

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ABSTRACT: We followed seasonal changes in the vertical distribution of protistan communities in Lake Biwa for 2 yr. Heterotrophic nanoflagellates (HNF) and plastidic nanoflagellates (PNF) were enumerated from different depths, and the seasonal community composition of protists was analyzed from the epilimnion and hypolimnion using 18S rRNA gene clone libraries. In the epilimnion, HNF were dominant during early summer and autumn, whereas PNF, which includes mixotrophs, had a higher contribution during the late summer and winter months. Changes in the abundance of HNF and PNF differed, and their peaks of abundance did not coincide, suggesting that these groups show contrasting responses to changes in environmental conditions. Protistan communities in the epilimnion and hypolimnion were represented mainly by cryptophytes and dinophytes, respectively, whereas cercozoans had a significant contribution in both (i.e. all libraries). We found high diversity and distinct protist communities in the epilimnion and hypolimnion, where the majority of the hypolimnion sequences had low similarity with related sequences in public databases. This indicates that little is known about protistan communities from deep freshwater lakes and that further studies from deep waters are needed to understand the role of these organisms.

KEY WORDS: PNF · HNF · Protist · Hypolimnion · Freshwater lakes · Clone library

INTRODUCTION

Protists are abundant and ubiquitous eukaryotic microorganisms in aquatic ecosystems, displaying substantial community diversity (Moreira & López-García 2002, Šlapeta et al. 2005). They play important roles in organic matter cycling by contributing to primary productivity through autotrophy and/or mixotrophy, becoming a major link between prokaryotes and higher trophic level organisms (Azam et al. 1983, Caron 1994, Nakano et al. 2001, Massana 2011) and recycling inorganic nutrients that limit algal growth (Caron et al. 1988, Nakano 1994a,b,c). The advancement of molecular techniques has given rise to an increase in diversity studies in recent years, which has led to the discovery of an unexpectedly high diversity of protists in various aquatic environments, especially oceans (López-García et al. 2001, Moon-van der Staay et al. 2001). By contrast, studies of protistan diversity from freshwater ecosystems are relatively limited (e.g. Charvet et al. 2012, Lepère et al. 2013, Taib et al. 2013). Freshwater ecosystems provide interesting habitats for ecological and protist diversity studies (Richards et al. 2005) because the environmental conditions of those systems are heterogeneous and are much more sensitive to external environmental variation than those in the oceans (Simon et al. 2015a).

Comparative studies have been conducted in various lakes to help us understand and compare protistan diversity. However, most of the studies from freshwater environments have been conducted mainly in shallow eutrophic lakes (Graham et al. 2004, Simon et al. 2015a,b) or lakes with anoxic hypolimnions (Lepère et al. 2006, Oikonomou et al. 2015, Lepère et al. 2016). In contrast, deep freshwater

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holomictic lakes have received less attention by researchers despite their global distribution. These lakes are an interesting environment for understanding seasonal community changes and dynamics of protists because of their water mixing patterns (Mukherjee et al. 2015). A few studies conducted from deep freshwater holomictic lakes have focused mainly on understanding the protistan communities of surface water, the site for active primary production (Medinger et al. 2010, Nolte et al. 2010). By contrast, hypolimnion waters, which have less primary production and biological abundance, have received less attention, and the information about abundance and composition of protists in the hypolimnion is still limited (Lepère et al. 2010, Tarbe et al. 2011, Fay et al. 2013, Mukherjee et al. 2015).

The ecological role of protists is directly linked to their community composition (Šimek et al. 1997). Studying the dynamics of protistan communities is, therefore, necessary to understand the community response to changes in biotic and abiotic parameters (Sherr et al. 2007, Lie et al. 2013, Kim et al. 2014). However, the seasonal changes in abundance and composition of protistan communities, together with their relationship with environmental and biological parameters in aquatic ecosystems, especially deep freshwater holomictic lakes, remain unclear (Carrias et al. 1998, Nolte et al. 2010). This is due to the lack of annual scale studies conducted from deep holomictic lakes because studies tend to be more focused on diversity analysis using molecular techniques from 1-time sampling or short-term seasonal sampling (Medinger et al. 2010, Nolte et al. 2010). Furthermore, the extent to which protistan communities show seasonal variation in deeper waters is still unknown because of the importance given only to surface waters.

To understand the annual dynamics and seasonal changes in protistan community composition of both surface and deep waters, the present study was conducted in Lake Biwa, a deep freshwater holomictic lake, for 2 yr. We aimed to reveal the seasonal changes in vertical abundance and composition of protists between the epilimnion and hypolimnion, using both microscopic and molecular techniques.

MATERIALS AND METHODS

Study site and sampling

The present study was conducted in Lake Biwa, which is the largest freshwater lake in Japan and has a surface area of 674 km^2 and a maximum depth of 104 m. The lake is monomictic and mesotrophic and has an oxygenated hypolimnion, where the annual minimum dissolved oxygen concentration is up to 4 mg l^{-1} (Kim et al. 2006). The water column of the lake is vertically mixed during winter (January to March) because of cooling and strong seasonal wind, whereas during the rest of the year (April to December), the water column is thermally stratified (Mukherjee et al. 2015).

Samples were collected for 2 yr (January 2012 to December 2013) on a monthly basis from Stn Ie-1 (35° 12′ 58″ N, 135° 59′ 55″ E), a long-term (since 1965) limnological survey station of the Center for Ecological Research, Kyoto University, Japan. The station has a maximum water depth of 73 m. The hydrographic structure was determined with a CTD profiler (SBE 911plus, Sea-Bird Electronics) equipped with an oxygen sensor (SBE 13 E, Sea-Bird Electronics). Water samples from 6 depths (5, 10, 15, 20, 50 and 70 m) representing the epilimnion, metalimnion and hypolimnion were collected with a 5 l Niskin sampler (General Oceanics). Samples were collected in clean plastic bottles, which were rinsed 3 times with sample water before collection and were kept cool and dark in an icebox. The samples were transported to the laboratory within 3 h of collection.

To determine the chl *a* concentration, 150 ml of the water sample was filtered through a GF/F filter (diameter 25 mm, Whatman) and analyzed following the *N*,*N*-dimethylformamide fluorometric method (Moran & Porath 1980, Mukherjee et al. 2015).

Total count of bacteria and nanoflagellates

Samples were fixed with a 1% final concentration of glutaraldehyde immediately after collection and stored at 4°C until filtration. One ml water samples were filtered through black polycarbonate membrane filters (pore size 0.2 µm, diameter 25 mm, Advantec) and stained with DAPI for enumeration of total bacteria (Porter & Feig 1980). Bacterial cells were visualized under UV light with an epifluorescence microscope (Olympus BX-50). Each sample preparation was counted twice at 1000× magnification from 20 randomly chosen fields (a minimum of 300 cells were counted).

For enumeration of heterotrophic nanoflagellates (HNF) and plastidic nanoflagellates (PNF), 30 ml of epilimnion and metalimnion (5 to 20 m) and 50 ml of

hypolimnion (50 and 70 m) waters were filtered through black polycarbonate membrane filters (pore size 0.8 µm, diameter 25 mm, Advantec) and stained with primulin (Caron 1983). HNF and PNF cells were observed with the epifluorescence microscope under UV and green excitation, respectively. For each sample, 100 randomly chosen fields were counted at 1000× magnification (a minimum of 100 cells were counted).

DNA extraction and clone library analysis

Samples for clone libraries were collected in May, August, October and December of 2012, corresponding to each season from the epilimnion (5 or 10 m), and in May (onset of stratification), August (stratified period) and December (before winter mixing) of 2013 from the hypolimnion (50 or 60 m) (Table 1). Water samples were collected after pre-filtering with a 20 µm mesh plankton net, and 1 to 2 l of the filtrate were filtered through polycarbonate membrane filters (pore size 0.8 µm, diameter 47 mm, Costar) at low vacuum (ca. 10 000 Pa) and stored at –30°C. DNA extraction was carried out with a PowerSoil DNA Isolation Kit (MO BIO Laboratories). 18S rRNA genes were PCR amplified by universal eukaryote primers EukA and EukB (Medlin et al. 1988). PCRs were performed in 20 µl of reaction volume with a Blend *Taq* PCR kit (Toyobo), and amplification was performed under the following conditions: initial denaturation at 95°C for 2 min, 35 cycles (95°C for 30 s, 59.5°C for 30 s, 72°C for 2 min) and final extension at 72°C for 7 min. PCR products were purified with the Exo-I and TSAP enzymes and cloned using a pT7 Blue Perfectly Blunt Cloning Kit (Novagen) according to the manufacturer's instructions.

Table 1. Details of the individual libraries. ME: May epilimnion; AE: August epilimnion; OE: October epilimnion; DE: December epilimnion; MH: May hypolimnion; AH: August hypolimnion; DH: December hypolimnion; OTUs: operational taxonomic units

Library	Month (season) and year	Depth (m)	No. of sequences analysed	No. of OTUs
ME	May (spring) 2012	5	70	40
AE	August (summer) 2012	10	169	73
OE	October (autumn) 2012	5	81	45
DE	December (winter) 2012	10	59	17
MH	May (spring) 2013	60	52	44
AH	August (summer) 2013	50	80	41
DH	December (winter) 2013	60	39	31

Sequencing of clones and phylogenetic analysis

Sequencing of clones was achieved by using the Euk 528F (Elwood et al. 1985) primer for the V4 region (Lovejoy et al. 2006) and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) to obtain an 800 to 900 bp segment of the gene. Sequencing was performed with an ABI Genetic Analyzer 3130xl Avant Capillary automated sequencer. The quality of the sequences was assessed by Sequencing Analysis Software v5.3.1 (Applied Biosystems), and the sequences were manually corrected and trimmed using MEGA6 software (Tamura et al. 2013). The closest match to each sequence was obtained from a DDBJ basic local alignment search tool (BLAST) search, and sequences with their closest matches under 97% were checked for chimeras with additional BLASTs of several sections of the sequence. Sequences were also checked for chimeras using mothur (Schloss et al. 2009) by reference-based chimera searches against the PR2 database (Guillou et al. 2013). Chimeras, low-quality sequences and sequences less than 400 bp were excluded from further analysis. The sequences were aligned using the CLUSTAL W package (Thompson et al. 1994). Operational taxonomic units (OTUs) were separated at 97% similarity, and a similarity matrix was calculated using Bioedit software (Hall 1999). Selected clones, representing 1 member of each OTU, were selected for bi-directional sequencing using EukA and EukB primers along with the internal forward and reverse primers 1055F (Holman et al. 2003) and D978 (Zimmermann et al. 2011). The sequences were deposited in the DDBJ nucleotide database under accession numbers AB996606 to AB996689 and LC165019 to LC165132. The rarefaction curves of clone libraries were plotted using the PAST program (Hammer et al. 2001).

Phylogenetic analysis of the epilimnion and hypolimnion protistan groups was conducted on clones from selected OTUs. Partial and full-length sequences were aligned using the SINA web aligner against the SILVA database (Pruesse et al. 2007). Maximum likelihood (ML) trees were constructed using MEGA6 software (Tamura et al. 2013) based on the appropriate model for individual data confirmed by the model test. Initial trees for the heuristic search were obtained by applying the neighbor-joining (NJ) method to a matrix of pairwise distances estimated using the maximum composite

likelihood (MCL) approach. ML trees were constructed from 1000 bootstrap replicates. To further confirm the results obtained from the ML trees, NJ analysis was performed using an MCL model with a 6-category Γ distribution of rate variation among sites. NJ trees were constructed from 2000 bootstrap replicates. The results of only ML trees were discussed because the topology of consensus NJ trees was the same as that of ML trees.

Statistics

Cluster analysis was conducted on a Bray-Curtis dissimilarity matrix based on square-root-transformed OTU abundance (number of reads per OTU), using the Vegan R package (Oksanen et al. 2013). A Spearman's rank correlation coefficient was calculated for testing the relationship between the abundance of HNF and PNF with environmental and biological parameters. A Wilcoxon signed rank test was conducted to compare the concentration and abundance of the biological parameters between the 2 yr. All statistical analyses were computed in the R environment (www.r-project.org) Average values are expressed as mean ± SD.

RESULTS

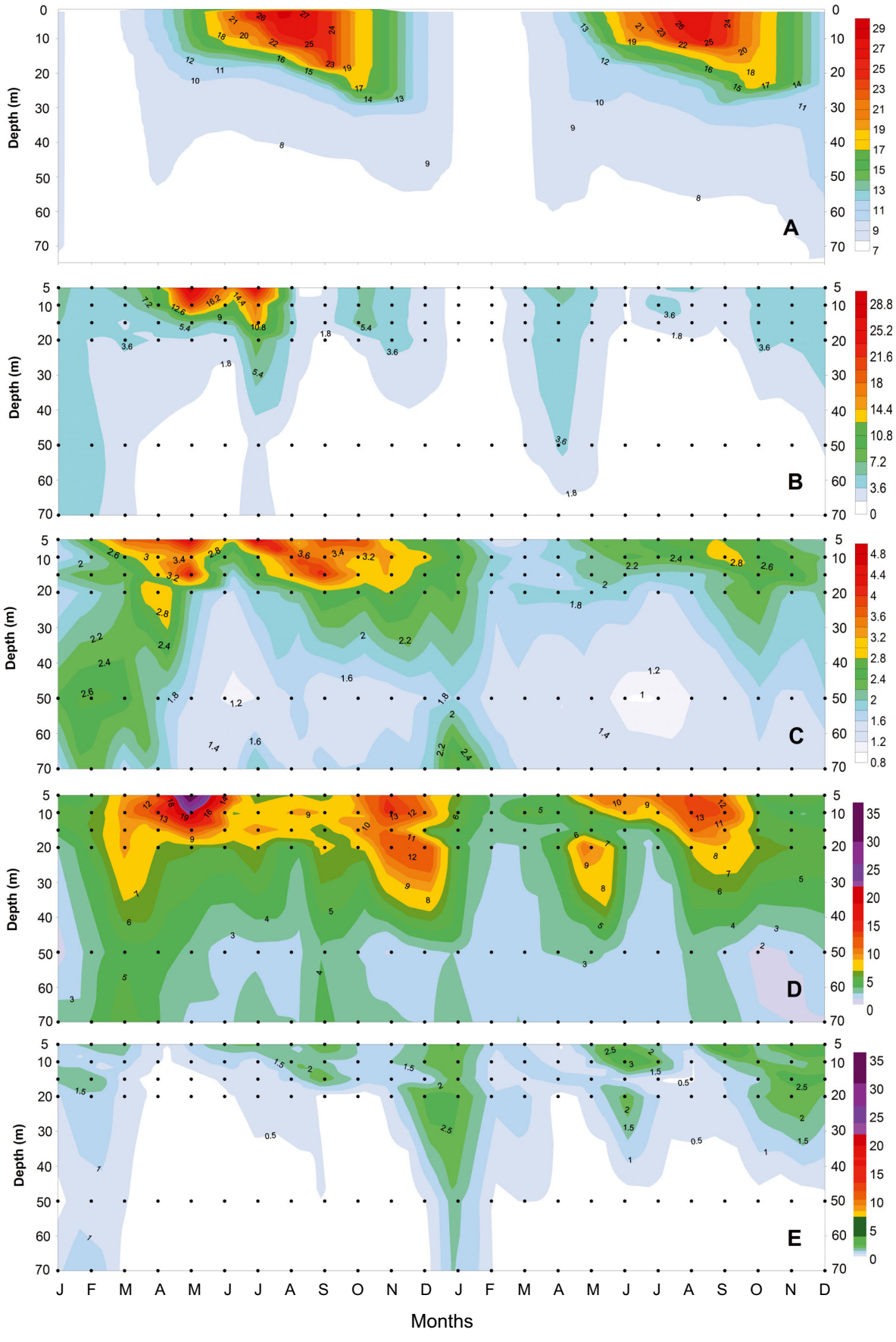
Physico-chemical and biological characteristics

The water column of Lake Biwa had a uniform temperature (average 7.6 ± 0.5°C) during the winter mixing (January to March) of 2012 (Fig. 1A). Thermal stratification commenced from April, with distinct stratification from May to November, where the thermocline was located at around 15 to 25 m. The temperature remained roughly constant in the hypolimnion (average 7.6 ± 0.3°C). Chl *a* concentration was uniform throughout the water column (average 4.4 ± 1.2 µg l^{-1}) during the mixing period of January to March. Chl *a* concentration in the epilimnion of 2012 showed distinct peaks in May and July, with the highest concentration in May at 5 m (31.1 µg l^{-1}) (Fig. 1B). However, in the hypolimnion, the concentration of chl *a* remained low throughout the year (average 0.8 ± 0.9 µg l^{-1}). The abundance of bacteria was relatively low throughout the water column during the mixing period (average 2.2 ± 0.5 × 10^6 cells ml^{-1}). Bacterial abundance in the epilimnion of 2012 followed 2 peaks, one during April and May and the other during July to October, with the highest abundance in July at 5 m (4.8 × 10^6 cells ml^{-1}) (Fig. 1C). However, bacterial abundance in the hypolimnion was relatively low (average 1.6 ± 0.3 × 10^6 cells ml^{-1}) and less variable.

The water column during the winter mixing of 2013 also had a uniform temperature (average 7.5 ± 0.2°C), which was followed by thermal stratification from April (Fig. 1A). Hypolimnion water of 2013 also had a roughly constant temperature (average 7.9 ± 0.7°C). Similarly, chl *a* concentration was uniform throughout the water column during winter (average 1.6 ± 1.0 µg l^{-1}) (Fig. 1B). Chl *a* concentrations in the epilimnion of 2013 showed peaks in April and July, with the highest concentration in April at 5 m (6.8 µg l^{-1}). Similar to the previous year, the concentration of chl *a* in the hypolimnion remained low (average 0.7 ± 1.0 µg l^{-1}). The abundance of bacteria was also relatively low throughout the water column during winter (average 1.9 ± 0.4 × 10^6 cells ml^{-1}) (Fig. 1C). Bacterial abundance in the epilimnion was high during August to October, with the highest abundance in September at 10 m (3.1 × 10^6 cells ml^{-1}). Likewise, bacterial abundance in the hypolimnion was relatively low (average 1.4 ± 0.2 × 10^6 cells ml^{-1}).

The changing patterns of the environmental and biological parameters in both years were similar. However, the concentration of chl *a* in the epilimnion of 2012 (6.5 ± 6.5 µg l^{-1}) was higher than that of 2013 (3.0 ± 1.6 µg l^{-1}) (Wilcoxon signed rank test, $p < 0.001$, $n = 96$). Similarly, the abundance of bacteria in the epilimnion was also higher in 2012 (2.8 ± 0.8 × 10^6 cells ml^{-1}) than in 2013 (2.1 ± 0.4 × 10^6 cells ml^{-1}) (Wilcoxon signed rank test, $p < 0.001$, $n = 96$). In contrast, in both years, the concentration of chl *a* and abundance of bacteria in the hypolimnion did not show a significant difference.

Fig. 1. Seasonal changes in vertical distribution of (A) temperature (°C), (B) concentration of chl *a* (µg l^{-1}), (C) abundance of bacteria (10^6 cells ml^{-1}), (D) abundance of heterotrophic nanoflagellates (HNF) (10^3 cells ml^{-1}) and (E) abundance of plastidic nanoflagellates (PNF) (10^3 cells ml^{-1}). Black dots represent sampling depth (in the case of temperature, the dots have been omitted to improve figure clarity due to high-resolution depth intervals by the CTD profiler). (D) and (E) are at the same scale to compare the abundance of HNF and PNF


Depth (m)
A
B
C
D
E
J F M A M J J A S O N D J F M A M J J A S O N D
Months

Dynamics of total HNF and PNF

The abundance of HNF was low throughout the water column (5.5 ± 2.5 × 10^3 cells ml^{-1}) during the winter mixing of 2012 (Fig. 1D). HNF were abundant in the epilimnion of 2012, with peaks during April and May and also in November, where their the highest abundance was in May at 5 m (36.9 × 10^3 cells ml^{-1}). In the hypolimnion, HNF abundance was low, with an average abundance of 3.2 ± 0.8 × 10^3 cells ml^{-1}. In contrast, PNF was abundant throughout the water column (1.2 ± 0.5 × 10^3 cells ml^{-1}) during the winter mixing of 2012 (Fig. 1E). The abundance of PNF was high in the epilimnion during June to September and also in December, just after the reduction of HNF abundance, with the highest abundance in December at 20 m (2.9 × 10^3 cells ml^{-1}). In the hypolimnion, the abundance of PNF remained low throughout the year (average 0.2 ± 0.1 × 10^3 cells ml^{-1}).

Similar to the previous year, the abundance of HNF was low throughout the water column (3.7 ± 1.2 × 10^3 cells ml^{-1}) during the winter mixing of 2013 (Fig. 1D). HNF became abundant in May and August, with the highest abundance in August at 10 m (13.9 × 10^3 cells ml^{-1}). In the hypolimnion, similar to the previous year, HNF abundance was low (average 2.6 ± 0.6 × 10^3 cells ml^{-1}). Similarly, PNF was abundant in the water column during winter mixing (average 1.3 ± 0.9 × 10^3 cells ml^{-1}) (Fig. 1E). High abundance was also recorded during June to July and September, just after the reduction of HNF abundance, with the highest abundance in September at 5 m (4.5 × 10^3 cells ml^{-1}). In the hypolimnion, as in the previous year, the abundance of PNF remained low (average 0.1 ± 0.1 × 10^3 cells ml^{-1}).

The average abundance of HNF in the epilimnion of 2013 (6.4 ± 3.3 × 10^3 cells ml^{-1}) was lower than that of 2012 (9.2 ± 5.6 × 10^3 cells ml^{-1}) (Wilcoxon signed rank test, $p < 0.05$, $n = 96$). In contrast, the average abundance of PNF in the epilimnion was higher in 2013 (1.6 ± 1.0 × 10^3 cells ml^{-1}) than in 2012 (1.2 ± 0.7 × 10^3 cells ml^{-1}) (Wilcoxon signed rank test, $p < 0.05$, $n = 96$). HNF and PNF showed contrasting dynamics, as their peak abundance never coincided (Fig. 1D,E). However, the abundance of PNF in the epilimnion was significantly lower than the abundance of HNF (Wilcoxon signed rank test, $p < 0.001$, $n = 192$). In the epilimnion, HNF abundance showed significant correlation with bacterial abundance, temperature and chl *a* (Table 2). However, both HNF and PNF abundances showed significant correlation with bacterial abundance and chl *a* in the hypolimnion, with similar ρ values.

Table 2. Results of Spearman's rank correlation (ρ) test between abundance of heterotrophic nanoflagellates (HNF) and plastidic nanoflagellates (PNF) with environmental and biological parameters. Separate correlation was conducted for the epilimnion (5 to 20 m) and hypolimnion (50 and 70 m) samples, as strong internal correlation among all the parameters was found from the full water column data (all depths)

Parameters	Epilimnion (ρ; p)	Hypolimnion (ρ; p)	All depths (ρ; p)
HNF and PNF	−0.02; 0.80	0.17; 0.26	0.50; <0.001
HNF and bacteria	0.60; <0.001	0.42; <0.05	0.75; <0.001
HNF and temperature	0.45; <0.001	−0.22; 0.13	0.61; <0.001
HNF and chl *a*	0.23; <0.05	0.48; <0.001	0.59; <0.001
PNF and bacteria	0.18; 0.07	0.37; <0.05	0.58; <0.001
PNF and temperature	0.16; 0.13	−0.06; 0.68	0.41; <0.001
PNF and chl *a*	−0.13; 0.21	0.49; <0.001	0.44; <0.001

Seasonal changes in protist communities

Seven clone libraries (4 from the epilimnion of 2012 and 3 from the hypolimnion of 2013) (Table 1) were analyzed, which yielded 550 clones representing 198 OTUs. Although various groups of protists were detected in the clone libraries, we focused mainly on the groups that include flagellated organisms to relate with the HNF and PNF abundances, as counted using the microscope (Fig. 1D,E). Rarefaction curves of the epilimnion and hypolimnion libraries did not reach an asymptote, except for the December epilimnion library (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a080p123_supp.pdf).

Cryptophyta dominated the epilimnion protistan community, with 48 and 29% of total sequence abundance and OTUs, respectively. Their sequence abundance and OTU contribution were the highest in May, accounting for 66 and 45%, respectively (Fig. 2). Five clones (M11, M5, O20, A10 and A9) clustered with the plastidic *Cryptomonas* sp. (Fig. 3A). However, clone M12 clustered with the heterotrophic freshwater CRY1 lineage and fell inside the freshwater cluster. Dinophyta was the most dominant group in the hypolimnion and was detected in all libraries (Fig. 2). The highest contribution of dinophytes was in August, accounting for 39% of total sequences and 24% of total OTUs. OTUs affiliated to Dinophyta were also obtained from the August and December epilimnion libraries, having the highest contribution in December, with 19% of total sequences (24% of total OTUs). Three clones from the

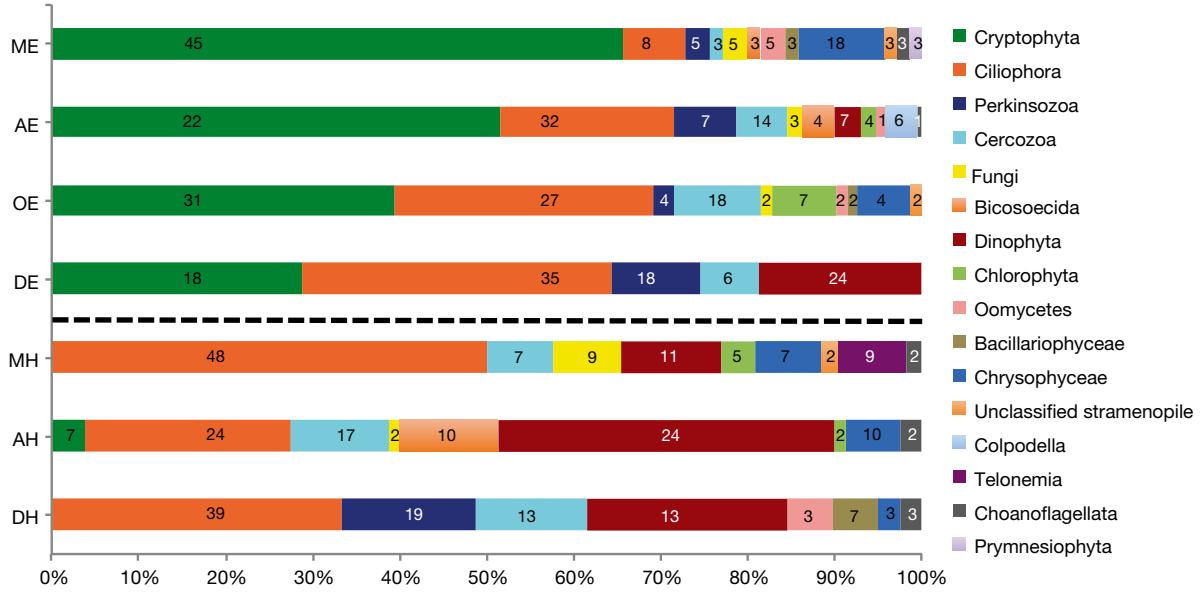


Fig. 2. Seasonal changes in community composition of protists by 18S rRNA gene clone library analysis using universal eukaryote primers, based on sequence abundance. The *x*-axis represents the group percentage of the total sequences per sample, and the *y*-axis represents the sample libraries. Numbers indicate the operational taxonomic unit (OTU) abundance of each group. A dashed horizontal line separates the epilimnion and hypolimnion libraries. ME: May epilimnion; AE: August epilimnion; OE: October epilimnion; DE: December epilimnion; MH: May hypolimnion; AH: August hypolimnion; DH: December hypolimnion

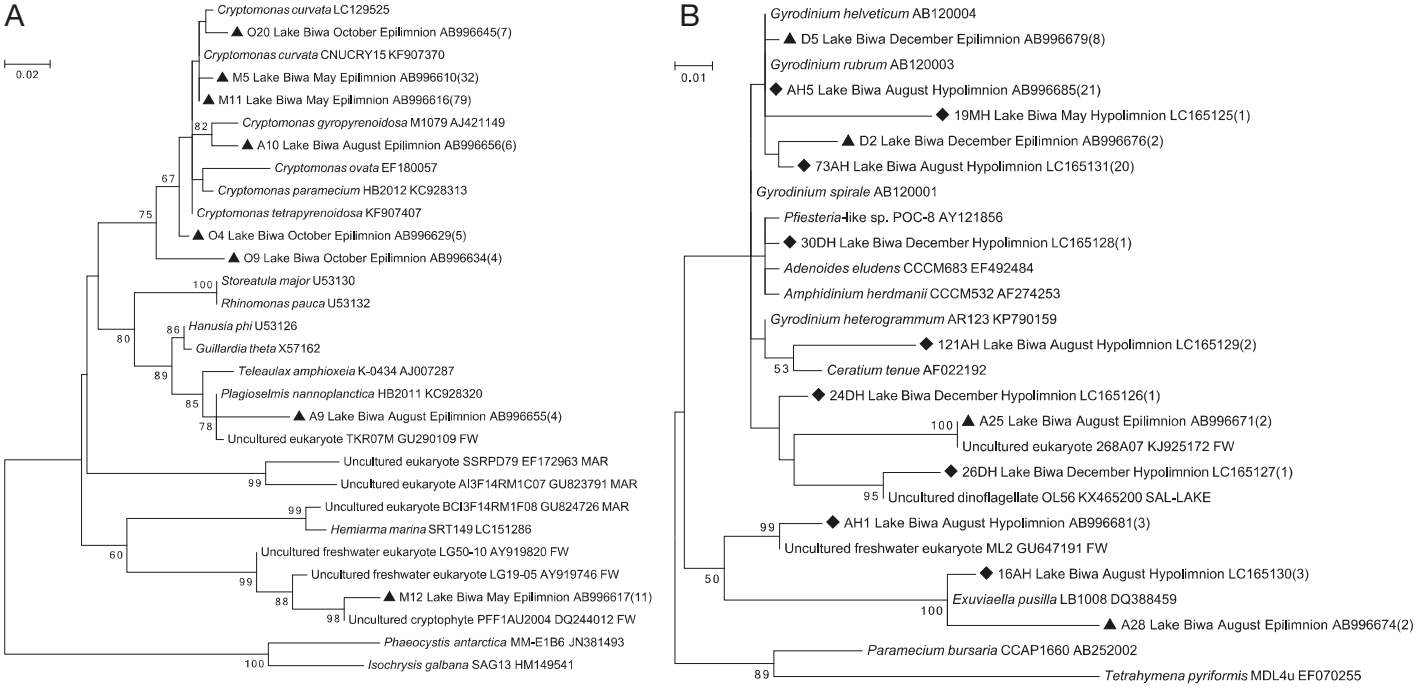


Fig. 3. Maximum likelihood (ML) phylogenetic tree using 18S rRNA gene sequences of (A) cryptophytes and (B) dinophytes. ML trees were calculated using the K2+Γ model of nucleotide substitution with a 6-category discrete approximation of a Γ distribution from 1000 bootstrap replicates. Bootstrap replicate support percentages ≥50% are shown next to the branches. Lake Biwa epilimnion clones are marked with black triangles, and hypolimnion clones are marked with black diamonds at the external nodes. Labels (FW: freshwater; MAR: marine; SAL-LAKE: saline lake) indicate the sources of uncultured sequences. The total number of sequences obtained from the particular operational taxonomic units is shown in parenthesis. Scale bar = 0.02 substitutions per site for (A) and 0.01 substitutions per site for (B)

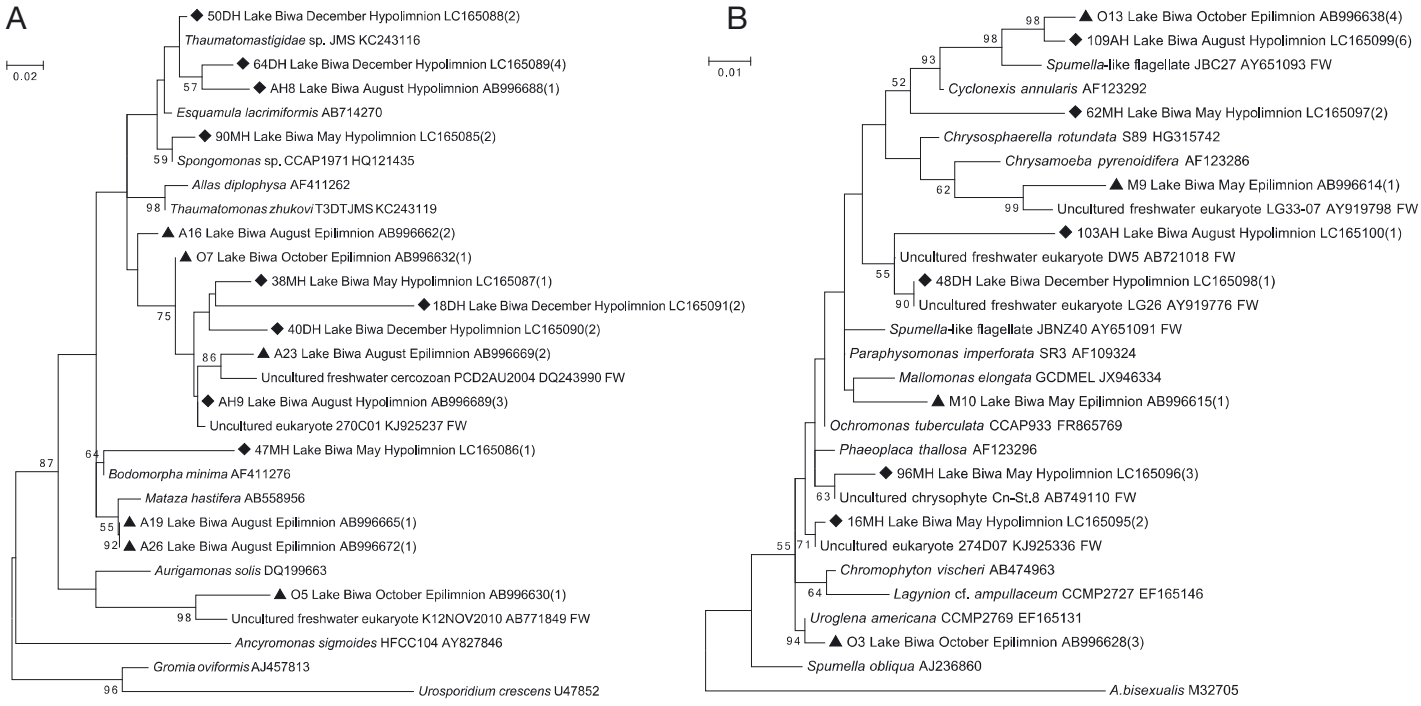


Fig. 4. Maximum likelihood phylogenetic tree using 18S rRNA gene sequences of (A) cercozoans using the K2+Γ model of nucleotide substitution (scale bar = 0.02 substitutions per site) and (B) chrysophytes using the T2+Γ+I model of nucleotide substitution (scale bar = 0.01 substitutions per site) (other details as in Fig. 3)

hypolimnion (AH5, 19MH and 73AH) and 2 from the epilimnion (D5 and D2) were related to *Gyrodinium rubrum* and *G. helveticum* (Fig. 3B). However, the hypolimnion clones were divergent from the other closely related sequences.

Cercozoans were present in all epilimnion and hypolimnion libraries, where their highest sequence abundance was in the October epilimnion, with a 10% contribution (18% of total OTUs) (Fig. 2). Some epilimnion clones (A23 and O5) had similarities to the other freshwater sequences (Fig. 4A). However, the majority of the hypolimnion clones (40DH, 38MH, 18DH, 64DH, AH8, 47MH) were divergent from the other sequences. Chrysophyceae were detected only in the May and October epilimnion libraries, representing 10% (18% of total OTUs) and 6% (4% of total OTUs) of total sequences, respectively. In contrast, chrysophytes were obtained from all hypolimnion libraries and contributed up to 6% of total sequences (10% of total OTUs) in August. The majority of the Lake Biwa clones from the epilimnion (O13, M9, O3) and hypolimnion (109AH, 48DH, 96MH, 16MH) clustered with freshwater species and sequences from other freshwater lakes (Fig. 4B). However, some of the hypolimnion clones (62MH, 103AH) were distantly related to other sequences. Perkinsozoa were present in all seasons in the epilimnion, with the highest diversity in December (10 and 18% of total sequences and OTUs, respectively). However, in the hypolimnion, perkinsozoans were observed only in December, with a relatively high contribution (15% of total sequences). The epilimnion and hypolimnion clones fell into 2 separate freshwater clusters and were related to the sequences obtained from other freshwater lakes (Fig. 5A). The Lake Biwa clones were divergent from the other sequences, where 2 epilimnion clones (D6 and A12) and 3 hypolimnion clones (7DH, 46DH and 4DH) formed separate sub-clusters. Bicosoecida were found in May and August epilimnion libraries, with 1 and 4% sequence contributions, respectively. Bicosoecids were present only in the August hypolimnion library, contributing 11% of total sequences (10% of total OTUs). Except for 1 epilimnion clone (A18), all the Lake Biwa bicosoecids were related to other freshwater environmental sequences (Fig. 5B). The hypolimnion clones (AH3 and AH6) were divergent from other sequences and formed a separate subcluster. Telonemia were only detected in the May hypolimnion library and accounted for 8% of total sequences. The Lake Biwa clones did not have close similarities to other freshwater telonemids (Fig. 5C), and the majority of clones (118MH, 93MH and 120MH) were divergent from other sequences. Cho-

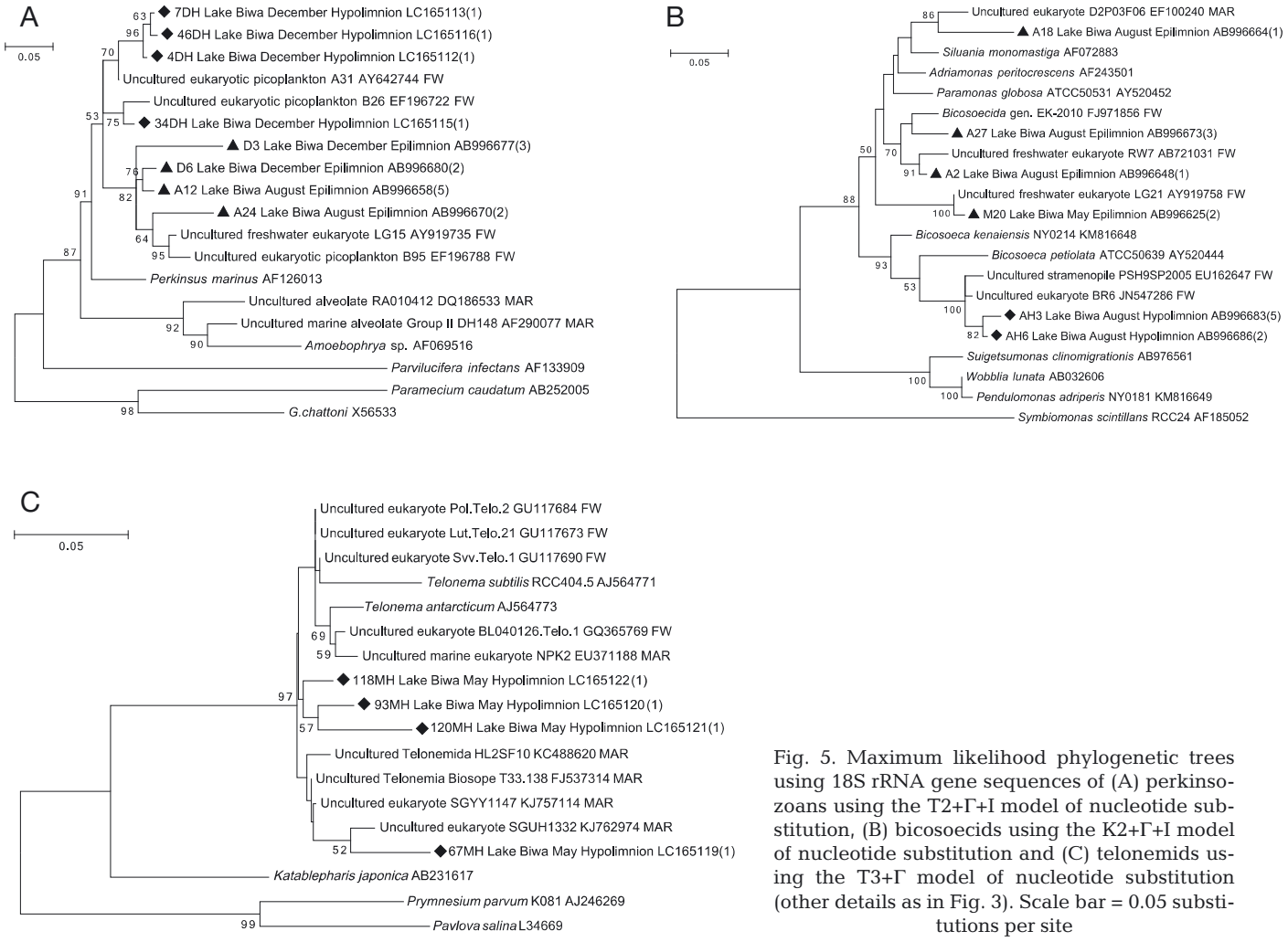


Fig. 5. Maximum likelihood phylogenetic trees using 18S rRNA gene sequences of (A) perkinsozoans using the T2+Γ+I model of nucleotide substitution, (B) bicosoecids using the K2+Γ+I model of nucleotide substitution and (C) telonemids using the T3+Γ model of nucleotide substitution (other details as in Fig. 3). Scale bar = 0.05 substitutions per site

anoflagellata were detected from May and August epilimnion libraries, with sequence contributions of 1 and 0.6%, respectively. Choanoflagellates were detected in all the hypolimnion libraries, where they contributed up to 3% of total sequences in December. Chlorophyceae were present in August and October epilimnion libraries, contributing up to 7% of total sequences. Chlorophytes were also detected from May and August hypolimnion libraries, contributing up to 4% of total sequences.

In the cluster analysis, except for the December epilimnion library, all the epilimnion and hypolimnion libraries were segregated, suggesting a significant difference between the epilimnion and hypolimnion communities (Fig. 6). Clustering of temperature and chl *a* also showed significant difference between the epilimnion and hypolimnion, and the segregation pattern was similar to that of the clone libraries (Fig. S2 in the Supplement).

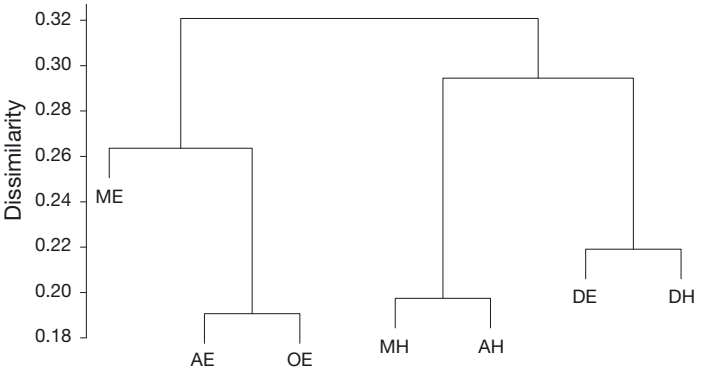


Fig. 6. Cluster dendrogram of the epilimnion and hypolimnion protistan communities based on Bray-Curtis dissimilarity matrix of square-root-transformed operational taxonomic unit abundance. ME: May epilimnion; AE: August epilimnion; OE: October epilimnion; MH: May hypolimnion; AH: August hypolimnion; DE: December epilimnion; DH: December hypolimnion

DISCUSSION

Seasonal shifts in the dominance of HNF and PNF

The average abundances of HNF and PNF in the epilimnion and hypolimnion of Lake Biwa were in the ranges observed in other studies in deep freshwater mesotrophic lakes (Mangot et al. 2009, Lepère et al. 2010). The abundance of HNF in the epilimnion of Lake Biwa with 2 seasonal peaks (Fig. 1D) corroborates other studies of freshwater lakes (Nakano et al. 1996, Carrias et al. 1998, Auer & Arndt 2001) because of the seasonal abundance of prey items (Salcher 2014). This is supported by the positive and significant correlation of HNF abundance with the bacterial abundance and concentration of chl *a* in the epilimnion (Table 2), which supports high densities of these bacterivorous (Sakka et al. 2000) and algivorous (Parslow et al. 1986) organisms (Fig. 2). Similarly, the high abundance of PNF during November to February (Fig. 1E) also corroborates other studies, as these autotrophic/mixotrophic flagellates dominate in freshwater lakes during winter (Sanders et al. 1989, 1990, Porter et al. 1996). Moreover, PNF in the present study were represented mainly by cryptophytes, which are generally abundant in oligo-mesotrophic lakes during winter (Dokulil & Skolaut 1986, Domaizon et al. 2003, Comte et al. 2006).

The 2 yr dynamics of HNF and PNF abundance have shown their respective dominance in different seasons in the epilimnion (Fig. 1D,E). HNF are known to dominate in the developmental phases of ecosystems during spring and autumn blooms (Auer & Arndt 2001, Mitra et al. 2014). In contrast, PNF are favored during summer, when the ecosystem is characterized by changes in nutrient conditions, increased particulate organic matter and decreased bacterial abundance due to high grazing (Mitra et al. 2014). Indeed, in Lake Biwa, a higher abundance of PNF was also recorded throughout the water column from November to February, when vertical mixing of the water column diluted bacterial abundance (Fig. 1C,E) and nutrient concentration increased. HNF are better competitors than PNF when bacterial abundance is high (Tittel et al. 2003, Mitra et al. 2014), whereas PNF can survive and dominate during low bacterial abundance because of their ability to utilize nutrients and light for their production (Tittel et al. 2003, Mitra et al. 2014, Caron 2016). Therefore, it is likely that the contrasting dynamics of HNF and PNF in Lake Biwa were due to the water mixing pattern and availability of resources in each season. Further studies from other lakes and laboratory experiments are needed to understand the reasons behind the contrasting dynamics of HNF and PNF in freshwater holomictic lakes.

Distinct protistan communities characterize the epilimnion and hypolimnion

In the present study, pre-filtration of the clone library samples with 20 µm mesh filters allowed us to remove larger cells, which might create biases in the molecular analysis due to the high copy number of their 18S rRNA gene. However, some larger cells might be included because of biases during the filtration processes (Nakano et al. 2001). Various groups of heterotrophic and plastidic protists were found in the epilimnion and hypolimnion of Lake Biwa, and rarefaction curves of nearly all the libraries did not reach saturation, suggesting incomplete sampling effort and high diversity (Fig. S1 in the Supplement).

The results of the present study are consistent with the seasonal studies in other freshwater lakes (Lepère et al. 2006, Nolte et al. 2010, Simon et al. 2015b), where despite having high diversity, only a few groups dominated the epilimnion protistan community. The epilimnion community was represented by both heterotrophic and plastidic groups like Cryptophyta, Ciliophora, Perkinsozoa and Cercozoa (Fig. 2), which is consistent with the results of other studies in freshwater lakes by cloning (Richards et al. 2005, Šlapeta et al. 2005, Chen et al. 2008, Lepère et al. 2008). The dominance of cryptophytes from all the epilimnion libraries (Fig. 2) agrees with the results of previous studies from other freshwater lakes (Carrias et al. 1996, Lepère et al. 2008, Simon et al. 2015a,b, Grossmann et al. 2016). Although most of the clones clustered with plastidic *Cryptomonas* sp., their divergence from other closely related sequences (Fig. 3A) is probably a result of gaps in the knowledge of cryptophytes from freshwater lakes (Nishino et al. 2015). Moreover, we found 1 OTU closely related to the heterotrophic CRY1 lineage (Shalchian-Tabrizi et al. 2008, Piwosz et al. 2016, Shiratori & Ishida 2016), which suggests that caution must be taken to assign phototrophy or mixotrophy in cryptophytes, as this lineage can contribute significantly to cryptophyte abundance (Piwosz et al. 2016).

Limited studies conducted from hypolimnion waters have shown the dominance of heterotrophic groups and the presence of putative parasites (Lepère et al. 2006, 2010). However, those studies were conducted at relatively shallow depths, and thus the communities in the deep waters are not properly

known. Complete light attenuation in Lake Biwa generally occurs above 30 m depth (Tsuda & Nakanishi 1989, 1990), thereby keeping the deep hypolimnion waters dark and possibly limiting the growth of phototrophs. Low abundance of PNF (Fig. 1E) along with the seldom-detected cryptophyte OTUs indicates the dominance of heterotrophic protists in the hypolimnion (Fig. 1D). Dinophytes dominated the hypolimnion protistan communities (Fig. 2), as the ecophysiological versatility of these flagellates enables them to represent a successful and abundant component of freshwater ecosystems (Smayda 2002, Graham et al. 2004, Taylor et al. 2008). Heterotrophy and adaptation to cold and low-light conditions (Chan 1978, Jakobsen et al. 2000, Charvet et al. 2012, Flaim et al. 2012, Oikonomou et al. 2015) are likely the reasons for their importance in the hypolimnion, as a majority of the dinophyte OTUs were closely related to *Gyrodinium rubrum* and *G. helveticum* (Fig. 3B), both being naked heterotrophic dinoflagellates (Takano & Horiguchi 2004). However, assessment of their actual abundance is needed because of the high copy number of 18S rRNA genes in dinophytes (Zhu et al. 2005, Potvin & Lovejoy 2009). Moreover, the feeding mode of several clones could not be deduced because of their divergence from cultured sequences and close relationship with the uncultured representatives.

Detection of cercozoans, a widespread omnivorous and parasitic group (Romari & Vaulot 2004, Piwosz & Pernthaler 2011), from all the epilimnion and hypolimnion libraries suggests their ecological importance in Lake Biwa throughout the year (Fig. 2). These results are consistent with the studies of French deep-water lakes, where cercozoans had a significant contribution in both epilimnion and hypolimnion waters (Lepère et al. 2006, 2010). Although the Lake Biwa epilimnion clones had similarities with sequences from other freshwater lakes (Fig. 4A), the distant relationship of the majority of the hypolimnion clones suggests that the cercozoans from the deep hypolimnion waters are significantly different.

Chrysophytes are a large group of flagellates (Lee et al. 2000), and the majority of their lineages inhabit freshwaters (del Campo & Massana 2011). Detection of chrysophytes from both the epilimnion and hypolimnion libraries (Fig. 2) suggests their ecological importance in the water column of Lake Biwa. The presence of chrysophytes in the epilimnion of May and October is probably due to their seasonal occurrence in freshwater lakes during spring (Kristiansen 1975) and autumn (Siver & Hamer 1992). The reason for their presence in all the hypolimnion libraries is not known. However, their ability to survive in cold and dark environments seems to be the most likely reason (Charvet et al. 2012). Elucidating the trophic role of these flagellates in Lake Biwa is difficult, as the majority of the epilimnion and hypolimnion chrysophytes had close relationships with the uncultured freshwater sequences (Fig. 4B).

The presence of perkinsozoans from all the epilimnion libraries and in the December hypolimnion library (Fig. 2) showed the importance of these putative parasites in Lake Biwa, similar to other freshwater lakes (Lepère et al. 2008, 2010, Mangot et al. 2013, Simon et al. 2015b). However, the divergence of hypolimnion clones suggests the presence of novel perkinsozoans in deep waters (Fig. 5A). Perkinsozoans are known to infect phytoplankton and bivalves in oceans (Burreson et al. 1994, Park et al. 2004), but their role in freshwater is not clearly understood (Mangot et al. 2011). Bicosoecids, a group of heterotrophic and attached flagellates, showed seasonal occurrence in both the epilimnion and hypolimnion. Bicosoecids are widely distributed in freshwaters (Richards et al. 2005, Šlapeta et al. 2005), where the sequences form a separate freshwater cluster (del Campo & Massana 2011). Although the majority of Lake Biwa bicosoecids fell into the freshwater clade, the divergence of hypolimnion clones (Fig. 5B) suggests that knowledge about the hypolimnion bicosoecids is still insufficient. The heterotrophic flagellate Telonemia, detected only from the hypolimnion (Fig. 2), has been reported mainly from marine waters (Shalchian-Tabrizi et al. 2007) and recently also from freshwater environments (Bråte et al. 2010, Triadó-Margarit & Casamayor 2012, Simon et al. 2015b). However, the distant relationship of the Lake Biwa telonemids with the other freshwater sequences (Fig. 5C) suggests the novelty of deep-water telonemids.

Although the hypolimnion samples were collected in the subsequent year, there was no significant difference in the environmental and biological parameters between the 2 yr in the hypolimnion (Fig. S2, Table S1 in the Supplement), suggesting the presence of similar communities in both years. The divergence of several hypolimnion clones from various groups with the environmental and cultured sequences in the public databases indicates the current lack of knowledge of deep-water protists. Moreover, distinct communities (Fig. 6) and the high diversity of hypolimnion protists (Fig. S1 in the Supplement) suggest that studies focusing only on the surface of deep lakes miss a large part of the diversity hidden in the deep waters.

CONCLUSIONS

The contrasting dynamics of HNF and PNF observed in the present study from the water column of Lake Biwa are probably due to water mixing and seasonal resource availability. Distinct protistan communities from the epilimnion and hypolimnion and the low similarity of several hypolimnion clones with the sequences in the database indicate the presence of novel protists in deep waters. High diversity and seasonal changes observed in the hypolimnion community highlight the need for seasonal analysis not only from the epilimnion but also from the deep hypolimnion waters to understand the role of these microbes in freshwater holomictic lakes.

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