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Alphaproteobacterial dominance in a large mesotrophic lake (Lake Biwa, Japan)

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ABSTRACT: We used fluorescence *in situ* hybridization to examine seasonal variation over 7 mo in the relative abundance of phylogenetic groups of bacterioplankton in the large (area 673.8 km^2, maximum depth 104 m) mesotrophic, freshwater Lake Biwa, Japan. *Alphaproteobacteria* dominated the community, with relative abundances (42 ± 12% [mean ± SD, n = 40] of total prokaryote abundance) much higher than those in other freshwater communities described in the literature. The second most abundant groups were *Actinobacteria* (14 ± 9.6%) and *Betaproteobacteria* (12 ± 6%) followed by the *Cytophaga-Flavobacteria* (CF) cluster (5.8 ± 3.5%). Filament communities were less diverse, consisting of only *Alphaproteobacteria* (64 ± 15% [n = 44] of the total filament abundance) and the CF cluster (28 ± 16%). Although seasonal patterns were different among different phylogenetic and morphotypic groups, *Alphaproteobacteria* consistently dominated the community throughout the seasons. Our data suggest that Lake Biwa differs from other lakes in some key aspects, urging further investigation of detailed taxonomic compositions and ecological features of freshwater *Alphaproteobacteria*.

KEY WORDS: *Alphaproteobacteria* · Bacterial community structure · Filamentous bacteria · Large lake · Lake Biwa · Fluorescence *in situ* hybridization

INTRODUCTION

Recent studies using molecular approaches have begun to reveal that there are indigenous communities of freshwater bacterioplankton which are different from soil and marine flora (Methé et al.1998, Zwart et al. 2002, Warnecke et al. 2004). The most abundant group in freshwater lakes usually belongs to *Betaproteobacteria* (Methé et al. 1998, Glöckner et al. 1999, Urbach et al. 2001), although *Actinobacteria*, *Bacteroidetes* (including the *Cytophaga-Flavobacter* [CF] cluster), and *Verrucomicrobia* also account for a significant fraction of freshwater communities (Glöckner et al. 2000, Urbach et al. 2001, Warnecke et al. 2005, Urbach et al. 2007). This typical bacterial community structure in freshwater lakes has been regarded as distinctive from that in marine waters where *Alphaproteobacteria* are often the most abundant group (Glöckner et al. 1999, Kirchman et al. 2005). Factors that may affect bacterial community structures in aquatic systems include the quality of dissolved organic matter (Crump et al. 2003, Kritzberg et al. 2006), trophic status (Yannarell & Triplett 2005), pH (Lindström et al. 2005, Schauer et al. 2005), temperature (Lindström et al. 2005), salinity (Wu et al. 2006), selective mortality (Weinbauer 2004, Pernthaler 2005), UV radiation (Warnecke et al. 2005) and immigrations (Lindström et al. 2005, 2006), although the relative importance of these factors in determining geographic patterns in bacterial community structure has yet to be clarified fully.

Several studies have documented seasonal variations in bacterial community structures in freshwater lakes (Pernthaler et al. 1998, Glöckner et al. 2000, Zwisler et al. 2003, Jardillier et al. 2004, Kent et at. 2004, Shade et al. 2007). These studies have revealed dynamic and, in some lakes, recurring features of communities (Schauer et al. 2006, Shade et al. 2007), which appear to be intricately influenced by both local and regional processes (Lindström et al. 2005,

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Yannarell & Triplett 2005). Thus, studies on bacterial biogeography in lakes should consider the extent of and mechanisms involved in seasonal variations of community structures. However, the information is still limited regarding controls of the seasonal dynamics of individual groups of bacterioplankton in freshwater lakes.

In lakes, filamentous bacteria can account for a substantial fraction (up to 80%) of total bacterial cell volume (Sommaruga & Psenner 1995, Pernthaler et al. 1998, Vrba et al. 2003), which has been interpreted as a consequence of selective elimination of rods and cocci over filaments during grazing by flagellates (Pernthaler et al. 1997, Jürgens et al. 1999, Šimek et al. 1999). Filamentous bacteria in lakes are usually accounted for by only a limited number of phylogenetic groups: typically by the CF cluster and to a lesser extent by *Betaproteobacteria* (Pernthaler et al. 1998, 2004, Schauer & Hahn 2005). Morphotypic features of bacteria may reflect not only phylogenetic variation but also phenotypic plasticity (Corno & Jürgens 2006). Few studies have examined community structures of filaments with concomitant measurement of environmental variables.

In the present study, we describe the relative abundance of major phylogenetic groups of bacterioplankton in the north basin of Lake Biwa, Japan, a large, mesotrophic basin with a water retention time of 5.5 yr. Large lakes represent important water resources globally (Herdendorf 1990) and have unique ecosystem features, e.g. the relative importance of internal cycling of materials as is the case in oceans (Tilzer 1990). However, data are limited regarding bacterial community structures in large lakes (Glöckner et al. 2000, Zwisler et al. 2003, De Wever et al. 2005), with only 1 paper, to our knowledge, being available on seasonal variations in the relative cell abundance of major phylogenetic groups (Zwisler et al. 2003). Consequently, bacterioplankton in large lakes are currently under-represented for considerations of bacterial biogeography. Our goal was to describe patterns in seasonal variations in the cell abundance of different phylogenetic and morphotypic groups of bacterioplankton in the pelagic site of Lake Biwa.

MATERIALS AND METHODS

Study site and sampling procedures. Water samples were collected at a pelagic station (35° 10.774′ N, 135° 56.661′ E; water depth, 57 m) in the north basin of Lake Biwa (Appendix 1, available as Supplementary Material online at www.int-res.com/articles/suppl/a048p231_app.pdf), a large (surface area, 674 km^2; water volume, 27.3 km^3; maximum depth, 104 m; watershed area, 3848 km^2; water retention time, 5.5 yr), tectonic, freshwater (average concentrations of Cl, Na and Ca are 7.5, 5.2 and 10.4 mg l^{-1}, respectively; Fujinaga et al. 2005) lake located in the central part of the Honshu Island, Japan (Horie 1984). The investigated area is mesotrophic; annual average concentrations of total phosphorus and chlorophyll *a* (chl *a*) concentrations in surface layers are 0.14 µM and 3.2 µg l^{-1}, respectively (Nishimura et al. 2005, Kim et al. 2006), with dissolved oxygen concentrations and pH varying in the ranges of 6.5 to 10.3 mg l^{-1} and 6.8 to 9.2, respectively, in the surface layer (for the period between 1990 and 2000; www.ecology.kyoto-u.ac.jp/biwako/teikan/index.htm). Depth profiles of water temperature and *in situ* chlorophyll fluorescence were obtained with a CTD probe (Sea-Bird Electronics, SBE-25). We used 5 l Niskin-X bottles (General Oceanics) to collect water samples near the surface (1 m) and in a subsurface layer within the euphotic zone. The subsurface depths corresponded to the layer of the chlorophyll maximum (3 to 15 m) when the fluorescence peak was clearly resolved (this was mostly the case between May and August). When the subsurface peak was not evident (on most sampling occasions between September and December), the subsurface samples were collected from the middle of the mixing layer (5 to 20 m). To collect zooplankton, a plankton net (mouth diameter, 15 cm; mesh size, 72 µm [Rigosha, NXX17]) was towed vertically within the epilimnion.

Fluorescent *in situ* hybridization (FISH) analyses of prokaryotes. Subsamples were fixed with paraformaldehyde solution (final conc. 2%). After fixation at 4°C for 24 h, prokaryotes were filtered onto 0.2 µm pore-size polycarbonate filters (Whatman) and stored at −20°C until analysis. We used rRNA targeted oligonucleotide probes following the hybridization conditions described in the literature (Cottrell & Kirchman 2004). Seven probes discriminate prokaryote groups at higher levels such as domain (Arch915 for Archaea and Eub338 for *Bacteria* according to Amann et al. [1995], but note that Pernthaler et al. [2002] have shown that Arch915 may hybridize to *Bacteria* in marine plankton) or division (or phylum) and subdivision (Alfa968 for *Alphaproteobacteria*, Beta42 for *Betaproteobacteria*, Gam42a for *Gammaproteobacteria*, and CF319 for the CF cluster according to Glöckner et al. [1999], and HGC69 for *Actinobacteria* according to Glöckner et al. [2000]). Unlabeled competitors were added for probing *Beta*- and *Gammaproteobacteria*. To resolve the CF cluster at a finer level, we used 3 probes (LD2-739 and CL500-653 according to Pernthaler et al. [2004] and R-FL615 according to Šimek et al. [2001]). Non338 (Glöckner et al. 1999) was used as a negative control.

We adopted a standard hybridization protocol according to Cottrell & Kirchman (2004) except that permeability of cell wall was enhanced with a lysozyme treatment (Pernthaler et al. 2002, Sekar et al. 2003). The sections of filters were dipped in low gelling point agarose (MetaPhor Agarose, Cambrex Bioproducts 50180), dissolved in Milli-Q water (Millipore, gradient A10) at a concentration of 0.2% (wt/vol), dried face up on parafilm (Menasha, PM-996) at 35°C and subsequently dehydrated in 96% (vol/vol) ethanol for 1 min at room temperature. The sections were incubated in a lysozyme (Sigma, L-7651) solution (10 mg ml^{-1} in 0.1 M Tris-HCl [pH 7.4, Wako, 015-20093] amended with 0.05 M ethylenediaminetetraacetic acid [EDTA, Sigma, E-1644]) at 37°C for 60 to 90 min, washed with Milli-Q water, dehydrated with 96% ethanol, dried at room temperature, and stored at –20°C until hybridization.

To capture cell images, a CCD camera (Hamamatsu Orca, C4742-95-12NR) was mounted on a fluorescence microscope (Olympus, BX60) equipped with a 100× UplanApo oil immersion objective and a 100 W mercury lamp. Cell images detected by the optics optimized for Cy3 (green excitation, Exciter HQ535/50x, Dichroic Q565LP, Emitter HQ610/75m) and DAPI (Exciter BP360-370, Dichroic DM400, Emitter BA420) were judged to be hybridized cells (probe-positives) using image analysis system (Media Cybernetics, Image Pro) following the algorithm of Cottrell & Kirchman (2004) with modifications to minimize effects of autofluorescence emitted from picocyanobacteria (see next paragraph) for prokaryotic cell counting. One pixel corresponded to 0.07 µm. The exposure time was manually optimized to maximize the cell detection with minimal background counts of hybridized cells obtained by the NON338 control probe. For each sample, 10 microscopic fields (1164.9 $µm^2$ per field) were examined. The number of DAPI-positive cells counted in 10 fields was 2600 ± 900 (mean ± SD). The cell volume of prokaryotes was determined semi-automatically as described in Cottrell & Kirchman (2004). Filaments, defined as cells with length >5 µm (Pernthaler et al. 2004), were counted manually using the images captured as described above. The number of filaments counted for each sample was 113 ± 148. The length and width of filaments were determined manually using ImagePro software (Media Cybernetics) to estimate the cell volume, with the assumption that the filaments were cylindrical.

In some samples, picocyanobacteria were highly abundant (up to 30% of total prokaryote abundance). In such cases, the precision of the estimates of the abundance of hybridized cells was largely compromised because of high background counts due to the autofluorescence of picocyanobacteria. To solve this problem, we modified Cottrell & Kirchman's (2004) algorithm of image analysis as follows. First, we defined that large cells (>0.44 $µm^2$ [100 pixels per cell] in area) captured with green excitation were picocyanobacteria and these cells were excluded from cell counts. We noted that the cell images were apparently larger than the actual cell size presumably because of the 'halo' ascribed to strong autofluorescence. Second, the cells located in the images (including the 'halo') of picocyanobacteria (as defined above) were excluded from the calculation of the ratio of hybridized cell counts to DAPI-positive counts. We failed to count Cy3-positive cells in 8 (total prokaryotes) or 4 (filaments) out of 48 samples because of interference by non-living particles.

Enumeration of viruses, nanoflagellates, phytoplankton, and mesozooplankton. Subsamples fixed with formaldehyde solution (final conc. 2%) were used to count viruses according to Noble & Fuhrman (1998), with slight modifications. Briefly, subsamples (0.3 to 1.0 ml) were filtered onto 0.02 µm pore size Anodisc filters (Whatman), stained with SYBR Green I (1:1000 dilution of the stock reagent, Molecular Probes, S-7563) for 20 min and mounted on slides with a mixture of immersion fluids (4:1 vol/vol of Citifluor and Vectashield). Virus-like particles were counted manually under an epifluorescence microscope (Exciter BP470-490, Dichroic DM505, Emitter BA510-550). Subsamples fixed with glutaraldehyde (final conc. 1%) were used for counting heterotrophic nanoflagellates (HNF) and *Uroglena americana*. Cells were collected on 0.8 µm pore size Nuclepore filters (Whatman), double stained with 4′,6-diamidino-2-phenylindole (DAPI; 2 µg ml^{-1}) and fluorescein isothiocyanate (FITC; 10 µg ml^{-1} dissolved in phosphate buffer, pH 7.2), and counted according to Sherr et al. (1993). Phytoplankton samples were fixed with Lugol's solution (final conc. 1%), concentrated by sedimentation, and counted under a light microscope (Olympus, BX50). Phytoplankton abundances were converted to carbon contents using conversion factors reported by Ichise et al. (1995). Mesozooplankton samples were fixed with 4% formalin (amended with 4% sucrose) and counted using an inverted microscope (Nikon, Optiphot-2).

Chemical analyses. Concentrations of chl *a* were determined fluorometrically using a fluorometer (Turner Designs, 10-AU) after extraction with 90% acetone (Wetzel & Likens 2000). Concentrations of NO_3^-, NO_2^-, and soluble reactive phosphorus (SRP) were determined spectrophotometrically using an autoanalyzer (Bran+Luebee, AACS II) according to the manufacturer's manual, whereas NH_4^+ concentration was determined by the OPA (*o*-phthaldialdehyde) method (Holmes et al. 1999). The sum of the concentrations of NO_3^-, NO_2^- and NH_4^+ are reported as the concentration of dissolved inorganic nitrogen (DIN).

RESULTS

Seasonal variations in the abundance of major phylogenetic groups of bacterioplankton

Seasonal variations in environmental parameters including temperature, nutrient concentrations and biotic variables are presented in Appendix 2 (available as Supplementary Material online at www.int-res.com/articles/suppl/a048p231_app.pdf). The abundance of prokaryotes varied in the range of 0.2×10^7 to 1.3×10^7 cells ml^{-1} (mean, $6.8 \times 10^6 \pm 2.4 \times 10^6$ cells ml^{-1}) (Fig. 1A). Eub338- and Arch915-positive cells accounted for 59.1 ± 8.8 and 8.3 ± 7.8% of the prokaryote community, respectively, with background counts (NON338 control) being 2.8 ± 1.3% (Table 1). Arch915-positive cells tended to be more abundant between May and July, with the maximum abundance of 36.4% in the subsurface layer on 13 June (Fig. 1C), whereas the abundance of Eub338-positive cells did not show a clear seasonal trend (Fig. 1B). The major fraction of prokaryotes was affiliated with 2 proteobacterial groups (*Alpha-* and *Betaproteobacteria*), the CF cluster, and *Actinobacteria*. *Alphaproteobacteria* persistently dominated the community, accounting for 41.7 ± 12.4% of total prokaryote abundance on average with the maximum (66.9%) on 17 July (Table 1, Fig. 2A). The second dominant groups were *Betaproteobacteria* and *Actinobacteria*, which contributed 11.8 ± 6.4 and 14.3 ± 9.6% of total cells, respectively (Table 1, Fig. 2B,D). *Actinobacteria* tended to be more abundant in the surface than the subsurface layer between May and mid-June, whereas this trend appeared to be reversed in summer (July to August) with greater relative abundances in the deeper layer (Fig. 2D). The CF cluster accounted for 5.8 ± 3.5% of total cells; the contribution tended to be high (>10%) in May (Table 1, Fig. 2C). *Gammaproteobacteria* were the least abundant group; their contribution (average, 2.2%) did not exceed the background level (Non338, 2.8%) (Table 1). The sum of the contributions of 4 phylogenetic groups (*Alphaproteobacteria*, *Betaproteobacteria*, the CF cluster, and *Actinobacteria*) was 73.6 ± 21.8%, which, after the subtraction of the negative control value, did not differ significantly from detection by the Eub338 probe (paired *t*-test, $p > 0.05$).

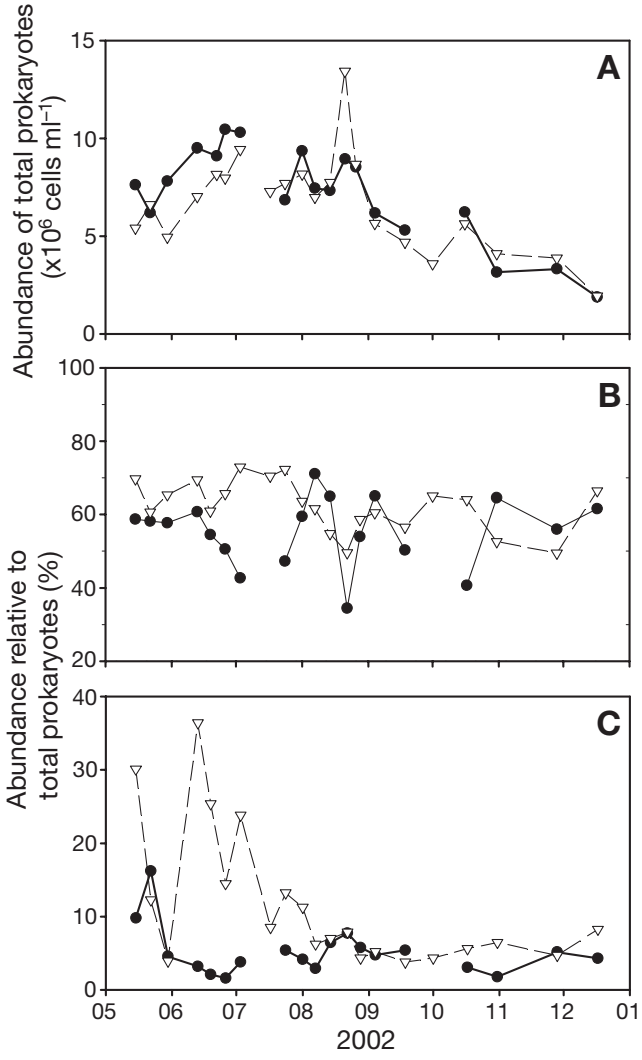

Fig. 1. Seasonal changes in (A) abundance of total prokaryotes, (B) relative abundance of EUB338-positive cells and (C) relative abundance of Arch915-positive cells. Data were collected at a depth of 1 m (●) and in subsurface layers (▽)

Relative abundances of the CF cluster were negatively correlated with the abundances of HNF ($r = -0.54$, $p < 0.001$), whereas the CF cluster and *Betaproteobacteria* were positively correlated with the cell abundances of the mixotrophic chrysophyte, *Uroglena americana* ($r = 0.66$, $p < 0.001$ for *Betaproteobacteria*, $r = 0.71$, $p < 0.001$ for the CF cluster) (Table 2). The contributions of *Chrysophyceae* to total phytoplankton biomass (%Chry) were positively correlated with the relative abundances of the CF cluster ($r = 0.73$, $p < 0.001$) and *Betaproteobacteria* ($r = 0.59$, $p < 0.001$) (Table 2).

Seasonal abundance and phylogenetic affiliations of filaments

The abundance of filaments was high during May and early July (Fig. 3A). The relative contributions of filaments to the total prokaryotic abundance and biovolume ranged from 0.02 to 1.4% (mean, 0.27 ± 0.30%) and 0.3 to 15.1% (mean, 3.2 ± 3.2%), respectively; the highest value was observed in the surface layer on 15 May (Fig. 3B). Total filament volume increased with increasing concentration of chl *a* ($r = 0.61$, $p < 0.001$), and *Uroglena americana* abundance ($r = 0.61$, $p < 0.001$) (Table 2). The contributions of filaments to total

Table 1. Summary of community structures of total and filamentous forms of prokaryotes determined by FISH in Lake Biwa. Negative control was not subtracted from the other results. CF cluster: *Cytophaga-Flavobacteria* cluster; CV: coefficient of variation

Phylogenetic group	Probe	% total prokaryotic abundance					
		Mean	SD	CV	Min.	Max.	n
Total							
Bacteria	Eub338	59.1	8.8	14.9	34.5	73.0	40
Alphaproteobacteria	Alfa968	41.7	12.4	29.7	20.1	66.9	40
Betaproteobacteria	Beta42	11.8	6.4	54.2	5.4	36.7	40
Gammaproteobacteria	Gam42a	2.2	1.6	72.7	0.0	6.8	40
CF cluster	CF319	5.8	3.5	60.3	1.6	18.1	40
Actinobacteria	HGC69a	14.3	9.6	67.1	2.7	38.4	40
Archaea	Arch915	8.3	7.8	94.0	1.6	36.4	40
Negative control	NON338	2.8	1.3	46.4	0.5	5.9	39
Sum of the major groups[a]		73.6	21.8				
Filament							
Bacteria	Eub338	87.6	6.2	7.1	69.7	94.5	44
Alphaproteobacteria	Alfa968	64.1	14.8	23.1	13.6	86.3	44
CF cluster	CF319	28.3	16.0	56.5	1.5	65.0	44
LD2 clade	LD2	17.0	14.1	82.9	0.6	53.6	44
Sum of *Alphaproteobacteria* + CF cluster		92.4	15.0				

[a]*Alphaproteobacteria*, *Betaproteobacteria*, CF cluster and *Actinobacteria*

prokaryotic biovolume were positively correlated with chl *a* ($r = 0.55$, $p < 0.001$), *U. americana* ($r = 0.76$, $p < 0.001$) and %Chry ($r = 0.59$, $p < 0.001$), whereas they were negatively correlated with the abundance of HNF ($r = -0.57$, $p < 0.001$) (Table 2)

Eub338-positive cells accounted for 87.6 ± 6.2% of the filaments, which were affiliated with only 2 major phylogenetic groups: *Alphaproteobacteria* and the CF cluster (Table 1). The relative abundances of these 2 groups displayed no clear seasonal trends (Fig. 3C). On average, filaments affiliated with *Alphaproteobacteria* and the CF cluster were 64.1 ± 14.8 and 28.3 ± 16.0% of total filament abundance, respectively (Table 1). Among 3 probes (LD2-739, CL500-653, R-FL615) used to resolve the CF cluster at a finer level, only the LD2-739 probe detected positive filaments. The LD2 clade on average accounted for 60.1% of total filamentous CF cluster and 17.0% of total filament abundance (Table 1, Fig. 3C).

DISCUSSION

The most striking finding of this study is that *Alphaproteobacteria* were the most abundant group of bacteria in Lake Biwa, which clearly differs from a general notion that they are less common in freshwater lakes (Glöckner et al. 1999, Kirchman et al. 2005) (Fig. 4, Appendix 3, available as Supplementary Material online at www.int-res.com/articles/suppl/a048p231_app.pdf).

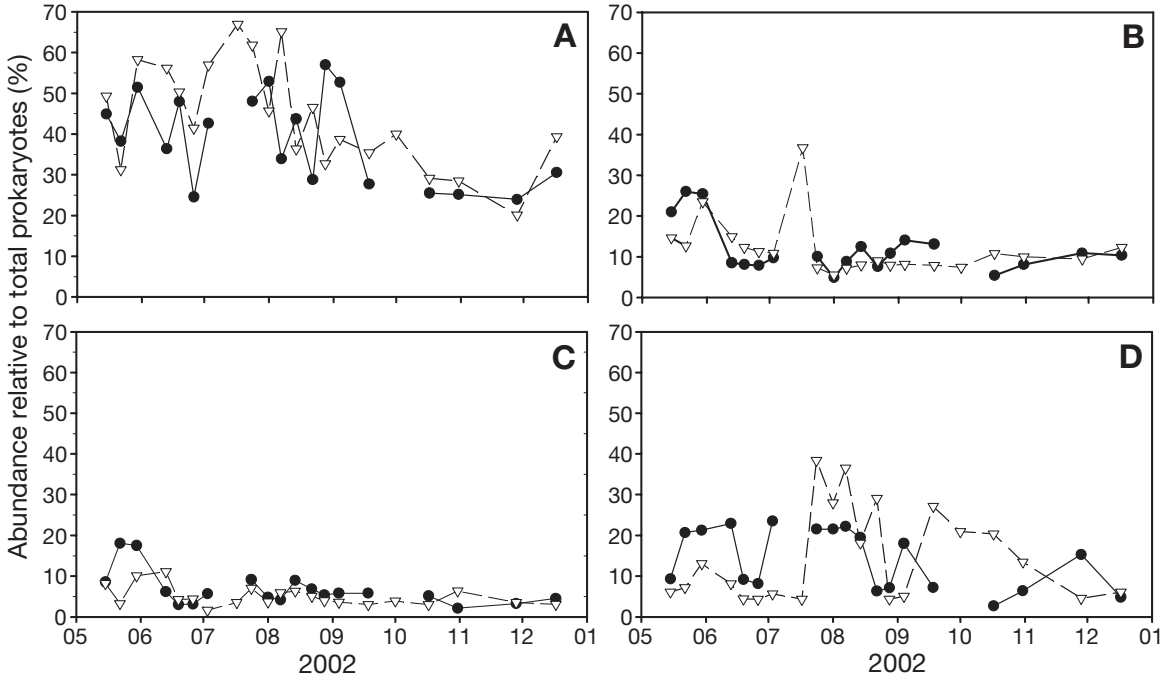

Fig. 2. Seasonal changes in relative abundance of (A) *Alphaproteobacteria*, (B) *Betaproteobacteria*, (C) the *Cytophaga-Flavobacteria* (CF) cluster, and (D) *Actinobacteria*. Data were collected at a depth of 1 m (●) and in subsurface layers (▽)

Table 2. Pearson's correlation coefficients between environmental variables and the relative abundances of different phylogenetic and morphotypic groups of bacteria (n = 40 – 44). α: *Alphaproteobacteria*; β: *Betaproteobacteria*; CF: the *Cytophaga-Flavobacteria* cluster; Actino: *Actinobacteria*; FV: filamentous biovolume; %FV: contributions of filaments to total prokaryote volume; Temp.: temperature; Chl *a*: chlorophyll *a* concentration; SRP: soluble reactive phosphorus; DIN: dissolved inorganic nitrogen; HNF: heterotrophic nanoflagellate abundance; *U.a.*: *Uroglena americana* abundance; %Chry: contribution of *Chrysophyceae* to total phytoplankton carbon biomass. Statistical parameters are presented for the pairs of variables with $p < 0.05$, but correlations are regarded significant only when $p < 0.001$ (in bold) after the Bonferroni adjustments for a 7 × 6 correlation matrix. For the correlation analysis, relative abundances of bacterial groups and %Chry and %FV were arcsine transformed, whereas concentrations of chl *a* and the abundances of HNF, *U. americana* and FV were log transformed

Group		Temp. (°C)	Chl *a*	SRP	DIN	HNF	*U.a.*	%Chry
α	r	0.32	0.32	−0.37	–	–	–	–
	p	0.044	0.043	0.018				
β	r	–	0.40	–	0.38	−0.41	**0.66**	**0.59**
	p		0.011		0.017	0.009	**<0.001**	**<0.001**
CF	r	–	0.47	–	–	**−0.54**	**0.71**	**0.73**
	p		0.002			**<0.001**	**<0.001**	**<0.001**
Actino	r	0.34	–	–	–	–	–	–
	p	0.034						
FV	r	–	**0.61**	–	–	−0.46	**0.61**	0.48
	p		**<0.001**			0.002	**<0.001**	0.001
%FV	r	−0.38	**0.55**	–	0.41	**−0.57**	**0.76**	**0.59**
	p	0.016	**<0.001**		0.009	**<0.001**	**<0.001**	**<0.001**

Indeed, *Alphaproteobacteria* consistently dominated the bacterial community, accounting for on average 42% of prokaryotic abundance. To our knowledge, there have been at least 2 studies reporting that *Alphaproteobacteria* were the most abundant group of bacteria in freshwater environments (Abe et al. 2003, Kirchman et al. 2004). Several studies have reported the presence of *Alphaproteobacteria* in freshwater environments on the basis of the data obtained by sequence analyses of 16S rRNA genes (Methé et al. 1998, Urbach et al. 2001, Lindström & Leskinen 2002), reverse line blot hybridization (Lindström & Leskinen 2002, Zwart et al. 2003, Lindström et al. 2005, Wu et al. 2006) and the identification of isolated strains (Bahr & Hobbie 1996). However, our data are the first that demonstrate, on the basis of seasonal coverage and cell counting by FISH, the persistent prevalence of *Alphaproteobacteria* in a freshwater lake. Comparisons of bacterial communities among lakes are complicated because of variations in the detection rates of bacterial groups owing to differences among studies in the protocol of FISH analysis; this hampers coherent examinations regarding factors controlling community structure across diverse lakes. Despite this limitation, Fig. 4 highlights the uniqueness of Lake Biwa communities with regard to the exceedingly high abundance of *Alphaproteobacteria* relative to other major groups.

Although previous studies have found that *Alphaproteobacteria* tend to become more abundant with increasing salinity in estuaries (Bouvier & del Giorgio 2002, Cottrell & Kirchman 2003, Kirchman et al. 2005) and high mountain lakes (salinity range, 0.02 to 22.3%; Wu et al. 2006), our data showing the alphaproteobacterial dominance in the freshwater body of Lake Biwa clearly indicates that salinity per se cannot generally explain the relative abundance of this group of bacteria. Some experimental studies have demonstrated a dynamic shift in bacterial community structures toward the dominance of *Alphaproteobacteria* in response to the enhancement of grazing pressures posed by bacterivorous flagellates (Pernthaler et al. 1997, Salcher et al. 2005). In Lake Biwa, however, grazing pressure alone probably cannot explain the dominance of *Alphaproteobacteria*, which were consistently abundant without any clear relationships with heterotrophic and mixotrophic flagellates.

Previous studies have reported some characteristic features in the occurrence of *Alphaproteobacteria* in freshwater environments. In northern Europe, Lindström et al. (2005) found that a freshwater clade of *Alphaproteobacteria* (LD12) was more abundant in lakes with longer retention time. In a freshwater delta in the western US, Stepanauskas et al. (2003) suggested that the abundance of T-RFLP fragments of *Alphaproteobacteria* was high during summer and autumn when the supply of phytoplankton-derived (autochthonous) dissolved organic matter is high. Analyses of 16S rDNA in lakes in the eastern US revealed increasing occurrence of *Alphaproteobacteria* with increasing lake size (Methé et al. 1998). The above results suggest that some freshwater *Alphaproteobacteria* are adapted to grow on autochthonous dissolved organic matter in systems with relatively long retention time and large water volume. However, other studies have suggested that there is no general tendency that *Alphaproteobacteria* become more abundant in larger lakes; *Alphaproteobacteria* were not detected (Lake Tanganyika, De Wever et al. 2005) or were less abundant (Lakes Constance [Zwisler et al. 2003] and Baikal [(Glöckner et al. 2000]) in large lakes in Africa and Eurasia (Appendix 3). Obviously, further studies are required to clarify the scale (local, regional and geographical) and nature (physical, chemical and biologi-

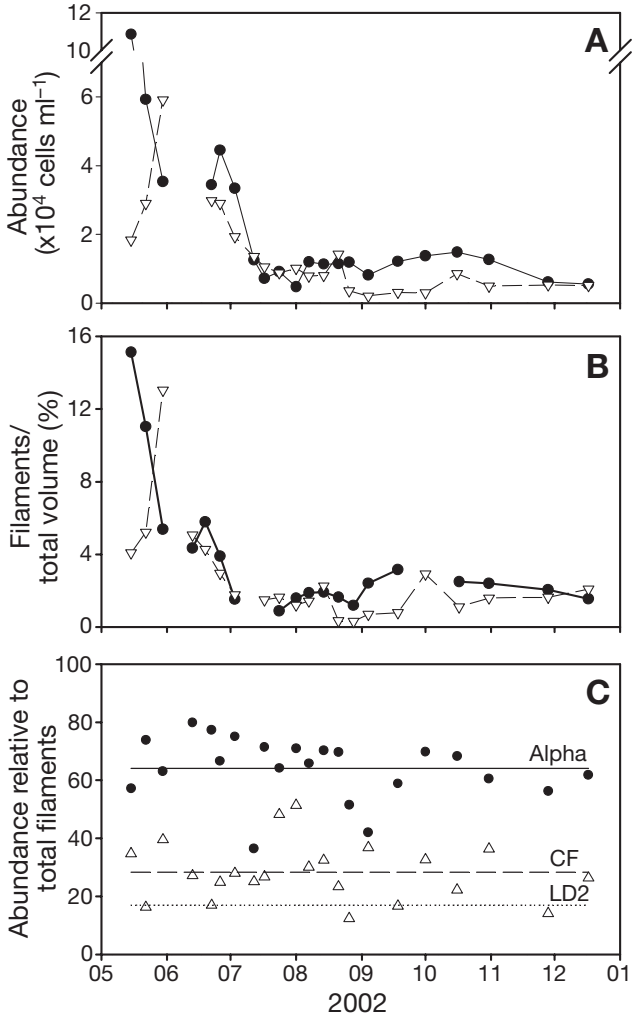

Fig. 3. Seasonal changes in (A) abundance of filamentous bacteria and (B) relative contribution of filaments to the total prokaryotic volume. Data were collected at a depth of 1 m (●) and in subsurface layers (∇). (C) Seasonal changes in the percentage contribution of filamentous forms of *Alphaproteobacteria* (●) and the CF cluster (Δ) to the total abundance of filaments. Each symbol represents the average value of that at the 2 water layers for each sampling date. Solid and dashed horizontal lines are the seasonal average values for *Alphaproteobacteria* and the CF cluster, respectively. Seasonal average value of the percentage contribution of the LD2 clade to total filament abundance is indicated by the dotted line

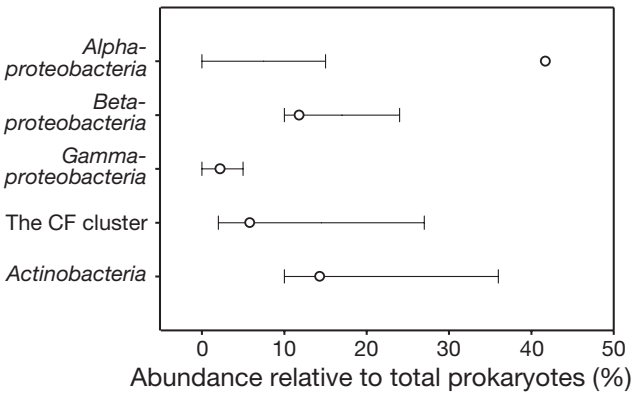

Fig. 4. Ranges reported in the literature of the mean relative abundances of the major phylogenetic groups of bacteria in freshwater lakes, ponds and reservoirs (original data are compiled in Appendix 3, available as Supplementary Material online at www.int-res.com/articles/suppl/a48p231_app.pdf). O: average abundances determined in Lake Biwa. Note that Lake Biwa largely differs from other lakes, although we do not exclude the possibility that this assertion will be eventually forced into revisions with the progress of studies given that the geographical coverage of the available data is limited especially in Asia (Appendix 3)

cal) of processes that account for the outstanding feature of the bacterial community; i.e. alphaproteobacterial dominance, in Lake Biwa.

Our results, which show that filaments were highly abundant during a bloom of mixotrophic flagellates, are consistent with observations in other lakes (Vrba et al. 2003, Pernthaler et al. 2004). Despite a large seasonal variation in the filament abundance, *Alphaproteobacteria* consistently dominated filamentous populations, with the second most abundant group being the CF cluster. This community structure of the filaments in Lake Biwa largely differs from previous observations in other lakes; the CF cluster, and to a lesser extent *Betaproteobacteria*, generally represent the major fraction of filaments (Pernthaler et al. 1998, 2004, Schauer & Hahn 2005). The LD2 clade of the CF cluster represented a significant fraction (17%) of total filaments in Lake Biwa, supporting the notion that this clade is cosmopolitan (Schauer et al. 2005).

The present study discriminated bacterioplankton at levels of division and subdivision, except for the CF cluster of filamentous bacteria. Each bacterial group distinguished at this high level of taxonomic unit consists of numerous phylotypes and species. Recent studies have suggested that ecological and physiological traits of bacteria are distinctive at finer taxonomic levels such as genus, species and even sub-species (Warnecke et al. 2004, Schauer & Hahn 2005) and that these 'ecotypes' display distinctive patterns in spatiotemporal variations (Schauer et al. 2005, 2006, Warnecke et al. 2005) that cannot be captured by the broad phylogenetic approach adopted in the present study. Nonetheless, studies have noted that there are systematic biogeographical patterns that can be distinguished at a broad phylogenetic level (Glöckner et al. 1999, Kirchman et al. 2005, Wu et al. 2006) and that different phylogenetic groups differ in patterns of use of dissolved organic matter (Cottrell & Kirchman 2000, Alonso & Pernthaler 2006), susceptibility to grazing (Pernthaler et al. 1997, Jezbera et al. 2005) or viral infection (Bouvier & del Giorgio 2007), and growth

(Zubkov et al. 2001, Yokokawa et al. 2004, Yokokawa & Nagata 2005). Our results for Lake Biwa support the hypothesis that different phylogenetic and morphotypic groups of bacteria differ in seasonal variation, presumably reflecting different traits and controls of individual groups. Our data also suggest that Lake Biwa differs from other lakes in some key aspects, although specific processes involved in community regulations remain to be identified in future studies. Regardless of the mechanisms, the dominance of *Alphaproteobacteria* during the investigation period of 7 mo suggests that this group of bacteria potentially plays a major role in ecosystem processes of Lake Biwa and other freshwater systems. We urge further investigation of detailed taxonomic compositions and ecological features (metabolism, growth and mortality) of freshwater *Alphaproteobacteria*, taking geography, limnology and biogeochemistry of lakes into consideration.

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