Ecology of bacterioplankton specific to the oxygenated hypolimnia of deep freshwater lakes

（大水深淡水湖の有酸素深水層に特有な細菌の生態解明）

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和文要旨（Abstract in Japanese）

海洋や湖沼の水中には 1 mL あたり約 10^6 細胞もの細菌が生息し、水域の物質循環や生態系において重要な役割を担っている。近年の分子生物学・シーケンス技術の急速な発展・普及に伴い、海洋や湖沼の表層、および深海においては、優占する細菌系統の存在が世界的に明らかになりつつあり、その生理・生態的な特徴づけや、生態系内における機能の解明が進められている。しかし、湖沼の深層、とりわけ全循環の大水深淡水湖で見られる有酸素深水層においては、その生物地球科学的な重要性が指摘されているにも関わらず、生息する細菌系統に関する知見がほとんど無く、細菌を介した物質循環プロセスに関してもほぼ未解明である。本研究では、大水深淡水湖の有酸素深水層に生息する細菌系統について統一的な知見を得ることを目的とした網羅的な調査を実施した。

本研究は次の 3 段階に分けて行った。まず、琵琶湖の有酸素深水層において CL500-11 系統の細菌が優占することを明らかにし、その時空間的な分布を、catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH)法によって追跡した（Chapter 2)。次に、16S rRNA 遺伝子アンプリコンシーケンスにより、琵琶湖沖における 3 水深×15 か月にわたる細菌群集組成を網羅的に調査し、深水層に出現する細菌系統の全体像を把握するとともに、表水層の細菌群集との比較を行った(Chapter 3)。最後に、琵琶湖で得た結果を他の湖で検証するため、日本全国の 10 の大水深淡水湖において調査を行い、CARD-FISH およびアンプリコンシーケンスを用いて表水層から深水層にかけて細菌群集組成を明らかにした(Chapter 4)。このように調査対象・研究手法を段階的に拡大し得られた情報を検証していくことで、有酸素深水層に生息する細菌群集の一般的な知見の構築を試みた。

その結果、Chapter 2 では *Chloroflexi* 門に属する CL500-11 系統の細菌の優占が琵琶湖全域の深水層で見られ、その現存量が全細菌比の最大 16.5%に及ぶことを明らかにした。さらに、CL500-11 は表水層および冬季の全層循環期にはほとんど出現しないことも明らかとなった。これにより、表層を対象としてきた従来研究では捉えられなかった、有酸素深水層のみに生息する細菌系統の存在が示された。Chapter 3 では、琵琶湖の水中に生息する細菌群集の全体像を初めて明らかにし、深水層には CL500-11 の他に、表水層ではほとんど見られない、*Planctomycetes* 門や *Thaumarchaeota* 門などに属する細菌系統

(CL500-3. CL500-15, CL500-37, MGI など)が生息することを明らかにした。一方表水層では、従来の研究で知られていたとおり、*Proteobacteria*, *Actinobacteria*, *Bacteroidetes* 門に属する細菌群が優占していた。この結果により、琵琶湖の有酸素深水層には、表水層と門レベルで異なる細菌群集が存在することが示された。さらに Chapter 4 では、琵琶湖で見つかったこれらの深水層特異的な細菌系統が、他の大水深淡水湖の深水層でも優占することを明らかにした。また、アンプリコンシーケンスで得られた塩基配列を 1 塩基レベルの違いに基づき分類する手法(oligotyping)により、全水層にかけて優占していた、既知の優占的な淡水性細菌系統（acI-B1, acI-A7 など）についても、深度や湖ごとに異なる亜集団が存在することを明らかにした。これらの結果より、有酸素深水層には表水層とは大きく異なる細菌系統群が存在することが複数湖において裏付けられ、その分布と現存量に関する統一的な知見を初めて得ることができた。

総じて、本研究により有酸素深水層に特異的な細菌系統群の存在が一般性・定量性をもって示された。この結果は、有酸素深水層の生態系および物質循環が表水層とは異なる独自の細菌群集によって駆動されていることを示唆しており、有酸素深水層に焦点を当てたさらなる研究の必要性を明示している。今後は、各細菌系統の生理・生態学的な特性を解明し、有酸素深水層の生態系・物質循環プロセスにおけるその役割と重要性を明らかにすることが課題である。

Abstract

Introduction

Approximately 10^6 cells of bacterioplankton live in a milliliter of ocean and lake water, playing important roles in the ecosystem and biogeochemical cycling in those environments. With the rapid development of molecular biology and sequencing technologies in recent years, the existences of ubiquitously dominant bacterioplankton lineages in marine and freshwater systems have been revealed. These predominant lineages are being studied eagerly for their ecophysiology, as their contribution to the global ecosystem and biogeochemical cycling should be significant.

The hypolimnion is a dark cold water layer that lies below the thermocline in a thermally stratified deep lake. In a holomictic oligo-mesotrophic lake, the hypolimnion remains oxygenated throughout a year, as the heterotrophic consumption of oxygen does not exceed the stock of hypolimnetic oxygen. The oxygenated hypolimnion accounts for a volumetrically significant part of the global freshwater systems, and the importance of biogeochemical processes in the realm driven by bacterioplankton have been suggested by numbers of studies. However, the predominant bacterioplankton lineages are unknown for the oxygenated hypolimnion, due to a limited number of studies. In the present thesis, I aimed to obtain a general overview of the bacterioplankton community inhabiting the oxygenated hypolimnion of deep freshwater lakes.

Materials and Methods

The present study was carried out by the following three stages. First, dominance of the CL500-11 lineage (phylum *Chloroflexi*) of bacterioplankton in the oxygenated hypolimnion of Lake Biwa (Japan) and their spatiotemporal distribution were revealed by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) (Chapter 2). The second, I performed amplicon sequencing of the 16S rRNA gene which comprehensively investigated bacterial community composition over 3 depths for 15 months at a pelagic site of Lake Biwa, and the members inhabiting the epilimnion and

hypolimnion were compared (Chapter 3). The third, in order to verify the observation in Lake Biwa, 10 deep freshwater lakes across Japan were investigated for the bacterial community composition from the epilimnion to hypolimnion using the CARD-FISH and amplicon sequencing techniques (Chapter 4). Finally, based on the results and data available in previous studies, a summarized overview of the bacterioplankton community inhabiting the oxygenated hypolimnion of deep freshwater lakes was built.

Results and Discussion

In Chapter 2, the quantitative investigation of the horizontal, vertical, and seasonal changes in the abundance of the CL500-11 lineage revealed that they dominated in the whole hypolimnion of Lake Biwa, and their contribution to the total bacteria reached a maximum of 16.5%. Meanwhile, they were virtually absent in the epilimnion and during the winter mixing period. These results revealed the existence of a bacterial lineage occurring exclusively in the oxygenated hypolimnion, which had never been to be detected by previous studies focusing only on epilimnion. In Chapter 3, the spatiotemporal distributions of bacterioplankton lineages in Lake Biwa revealed that, other than CL500-11, members affiliated with the phyla *Planctomycetes* and *Thaumarchaeota* (e.g., CL500-3, CL500-15, CL500-37, and MGI) were also hypolimnion-specific lineages. On the other hand, members occurred in the epilimnion were well-studied ubiquitous freshwater lineages affiliated with the phyla *Actinobacteria*, *Proteobacteria* and *Bacteroidetes*. These results indicated that the epilimnion and hypolimnion of Lake Biwa were inhabited by different bacterial communities at the phylum level. In Chapter 4, it was confirmed that the hypolimnion-specific lineages found in Lake Biwa also dominated in other deep freshwater lakes. In addition, an analysis of single-nucleotide variations in partial 16S rRNA gene sequences (oligotyping) suggested the presence of different subpopulations among the lakes and water layers for lineages occurring in the entire water column (e.g., acI-B1 and acI-A7). These results collectively support the existence of hypolimnion-specific bacterioplankton that are ubiquitous and significantly different from epilimnetic ones and provided comprehensive

information on their distribution and abundance.

Conclusion

This study provided the first general overview of the bacterioplankton community in the oxygenated hypolimnion of deep freshwater lakes. The results suggest that the ecosystem and biogeochemical cycling in oxygenated hypolimnion are driven by the specific bacterial community significantly different from that in epilimnion. It evidently proposes the needs for future works investigating the ecophysiological characteristics of individual bacterial lineages to understand their roles in the ecosystem and biogeochemical processes in deep freshwater lakes.

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Chapter 1: General introduction

Bacterioplankton are defined as free-living bacteria and archaea in the water column of aquatic environments. They ubiquitously inhabit marine and freshwater systems, with a typical order of magnitude of 10^6 cells mL^{-1} (Kirchman, 2012). Bacterioplankton play important roles in the ecosystems, as they consume dissolved organic matter (DOM) which is generally the most abundant source of organic carbon in water column, and then they transfer the incorporated carbon to higher trophic levels via protistan grazing (the microbial loop theory; Azam et al., 1983). Despite their small cell size, bacterioplankton are the most significant organisms in terms of biomass, driving the primary fluxes of energy and materials in aquatic ecosystems (Pomeroy et al., 2007). With the recent development of molecular and sequencing technologies, ecological studies on bacterioplankton have been attracting many researchers and leading the field of microbial ecology in terms of both quality and quantity (Pernthaler 2005; Newton et al., 2011; Karl and Church 2014; Fuhrman et al., 2015).

As >99% of bacteria inhabiting natural environments elude cultivation (Puspita et al., 2012), various cultivation-independent techniques based on 16S rRNA gene sequence phylogeny have been developed to investigate bacterial community composition. Among them, fluorescent *in situ* hybridization (FISH) (Pernthaler et al., 2004; Amann and Fuchs, 2008) is a powerful tool, since it allows direct detection of cells of a target lineage and facilitates accurate quantification of their abundance (Allgaier and Grossart, 2006a; Salcher et al., 2008). However, FISH can target only bacterial members for which a specific probe is available, meaning that it cannot detect lineages without 16S rRNA gene sequence information in advance. For comprehensive retrieval of 16S rRNA gene sequences from a natural bacterial assemblage, cloning-sequencing of the gene amplified by bacterial universal primers is a common technique. Although this technique is less quantitative than FISH, it can reveal the whole community diversity including previously unknown lineages (Giovannoni et al., 1990; Glöckner et al., 2000). Recently,

moreover, direct amplicon sequencing using a high-throughput sequencer have become a more general approach to replace the cloning-sequencing technique (Caporaso et al., 2011; Eiler et al., 2012), as it has increased the throughput more than 10,000 folds.

As research progresses, there are growing consensuses for predominant bacterioplankton lineages in each aquatic system. For instance, SAR11 is well-known for a predominant lineage in the global surface ocean (Morris et al., 2002), while the SAR202 and MGI lineages are known to dominate in the deep oceans (Schattenhofer et al., 2009). In freshwater systems, members of the phyla *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* (e.g., acI, LD12, *Polynucleobacter*, and *Limnohabitans*) are commonly known predominant lineages (Zwart et al., 2002; Newton et al., 2011). These ubiquitous and abundant lineages are being studied eagerly for their ecophysiology, as their contribution to the global ecosystem and biogeochemical cycling may be high.

The consensus on predominant freshwater bacterioplankton is based on studies in surface water layers (epilimnion), however, deeper layers (hypolimnion) have been paid much less attention by researchers. The hypolimnion is the dark cold water layer that lies below the thermocline in a thermally stratified deep lake. In a holomictic (i.e., the water layers are not permanently stratified but mixed seasonally) oligo-mesotrophic lake, the hypolimnion remains oxygenated throughout the year, since heterotrophic consumption of oxygen does not exceed the stock of hypolimnetic oxygen. Although the oxygenated hypolimnion occurs in a limited number of lakes, it is a volumetrically significant realm due to its vertical expanse. For instance, Lake Baikal and the Laurentian Great Lakes together hold 36.6% of the freshwater in the global lakes (Tilzer and Serruya, 1990), and a majority of the water is kept in the oxygenated hypolimnion during stratification, as the average depths of Lake Baikal (740 m) and the Laurentian Great Lakes (53–148 m; except for Lake Erie = 18 m) are much deeper than the depth of the upper mixing layer (epilimnion: generally <20 m; Mazumder and Taylor, 1994). In such a lake, the hypolimnion takes important ecological role by accumulating and remineralizing organic matter derived from surface production and regenerate nutrients that may then be supplied to the epilimnion through winter vertical mixing (Wetzel,

2001). Recent studies have suggested that DOM in oxygenated hypolimnion is enriched by the slowly consumed semi-labile fraction (Maki et al., 2010) and can be transformed into a more recalcitrant form by microbes (Thottathil et al., 2013; Hayakawa et al., 2016), as shown by the microbial carbon pump theory proposed for the ocean (Ogawa et al., 2001; Yamashita and Tanoue, 2008; Jiao and Zheng, 2011; Hansell, 2013). Other studies have demonstrated that nitrification (Small et al., 2013), dark carbon fixation (Callieri et al., 2014), and methane oxidation (Murase and Sugimoto, 2005; Bornemann et al., 2016) are also present in the oxygenated hypolimnion. The bacterioplankton inhabiting this realm thus are responsible for these important biogeochemical processes, meaning that their ecophysiological characteristics should be studied.

To date, only few studies have reported bacterioplankton community composition in oxygenated hypolimnion. Among them, studies at Crater Lake, USA (maximum depth = 589 m) have performed the most comprehensive investigation (Urbach et al., 2001, 2007). In the studies, the bacterioplankton community composition from the epilimnion to the oxygenated hypolimnion is determined by a cloning-sequencing analysis, and the vertical distribution patterns of several lineages are followed by semi-qualitative dot-blot hybridization targeting 16S rRNA gene sequence. The results indicate that the oxygenated hypolimnion of Crater Lake was dominated by "unusual" bacterioplankton lineages, which have not been reported from other freshwater systems. The authors concluded that the dominance of unusual bacterioplankton community could be attributable to unique physiochemical properties of Crater Lake: the extremely low concentrations of DOM, nitrogen, and trace metals; hydrothermal influx to the lake; and high UV light penetration due to the ultra-oligotrophic clear water. To verify their assumptions, investigations in other deep freshwater lakes and comparison of the results between the lakes are required. However, there have been no consensus on predominant lineages in oxygenated hypolimnion, because the studies still remain scarce.

In the present thesis, I aimed to summarize the first comprehensive overview on predominant bacterioplankton lineages inhabiting the oxygenated hypolimnion of

deep freshwater lakes, by investigating their diversity, ubiquity and abundance. The work consists of three chapters (Fig. 1-1). In the following Chapter 2, the dominance and spatiotemporal distribution of a hypolimnion-specific bacterioplankton lineage, CL500-11, were revealed by FISH in Lake Biwa, Japan. The result then motivated the study in Chapter 3, which investigated the spatiotemporal distributions of individual bacterioplankton lineages in Lake Biwa using a high-throughput amplicon sequencing. The data indicated the presence of several hypolimnion-specific lineages in addition to CL500-11. To verify the observations in Lake Biwa, the research in Chapter 4 expanded the study site to 10 deep freshwater lakes across Japan and performed both FISH and amplicon sequencing. Finally, by reviewing the results and data available in previous studies, I proposed a general overview of bacterioplankton lineages inhabiting oxygenated hypolimnion and reached a conclusion that the ecosystem and biogeochemical cycling in oxygenated hypolimnion are driven by specific bacterioplankton lineages that are significantly different from those in epilimnion.

Tables and Figures

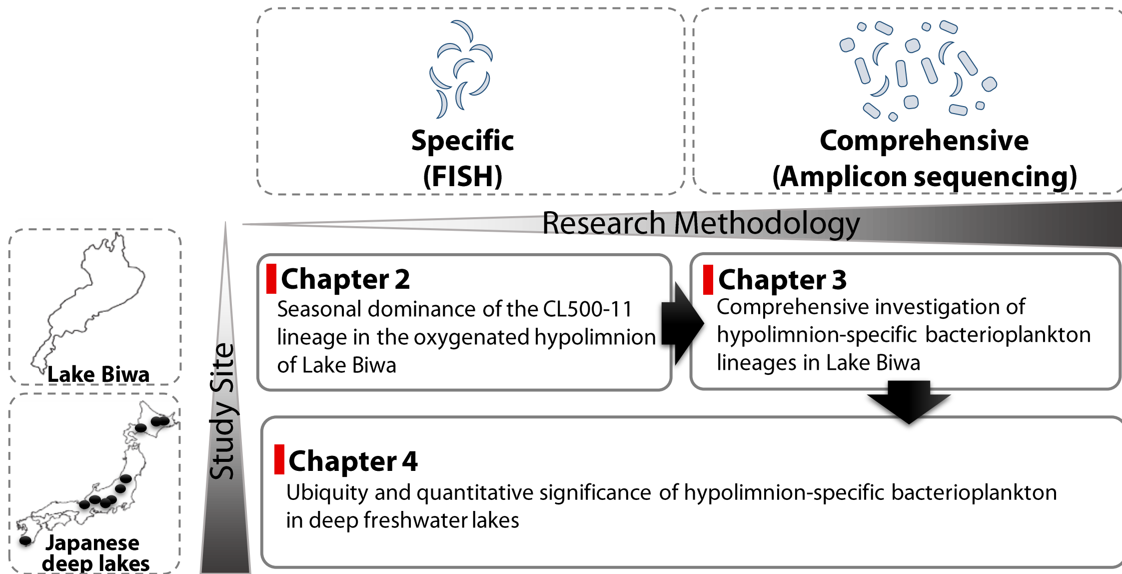


Figure 1-1

Overview of the three main chapters of the thesis. The present study was started by specifically targeting the CL500-11 lineage in Lake Biwa (Chapter 2). This result motivated to perform more comprehensive investigation on the hypolimnetic bacterioplankton community in Lake Biwa (Chapter 3). Finally, the study was expanded to 10 Japanese deep freshwater lakes to verify the generality of the conclusion in previous chapters (Chapter 4).

Chapter 2: Seasonal dominance of the CL500-11 lineage in the oxygenated hypolimnion of Lake Biwa

Introduction

Planktonic bacteria are the most abundant and active organisms in the pelagic zones of lakes. The recent development of molecular tools has allowed researchers to identify bacterial communities as assemblages of functionally diverse phylogenetic groups, and current study trends are now directed toward the characterization of each group (Hahn et al., 2005; Newton et al., 2007; Watanabe et al., 2009; Šimek et al., 2010). Some studies have demonstrated cyclic dominances of particular bacterial groups. For instance, it has been reported that *Polynucleobacter* (*Betaproteobacteria*) and LD12 clade (*Alphaproteobacteria*) are dominant, respectively, in the epilimnion in Lake Mondsee (Wu and Hahn, 2006b) and in prealpine lakes (Salcher et al., 2011a) during the summer stratification period. It is likely that each bacterial group occupies a particular ecological niche and dominates in response to transitions in niche availability. As the oxygenated hypolimnion consists of an isolated water mass during the stratification period, it may provide a distinct ecological niche to be inhabited by bacterioplankton adapted to this environment. Nevertheless, information on the dominant phylotypes in the oxygenated hypolimnion remains scarce, in contrast to the extensively explored epilimnion.

The CL500-11 cluster of the phylum *Chloroflexi* was described by Urbach et al. (2001) as a predominant group of bacterioplankton in the oxygenated hypolimnion of the freshwater ultraoligotrophic Crater Lake, USA. Quantitative rRNA-targeted dot-blot hybridization indicated that CL500-11 represented around 50% of the nonplastid bacterial rRNA in the hypolimnion during the summer stratification period (Urbach et al., 2001, 2007), suggesting that the cluster is a potential player in the process of hypolimnetic mineralization. However, CL500-11 dominance has been reported only from Crater Lake to date, and this is the single known case of *Chloroflexi* dominance in

an oxygenated freshwater system. A growing consensus on major bacterial groups in oxygenated freshwaters indicates that the phyla *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* are generally the predominant groups of bacterioplankton, irrespective of trophic status or local climate (Zwart et al., 2002; Newton et al., 2011). Therefore, *Chloroflexi* CL500-11 bacteria have been regarded as an unusual phylotype whose ecological characteristics remain obscure because of lack of information.

Here, I report on the identification of a second case of CL500-11 dominance in the oxygenated hypolimnion, in this case, from Lake Biwa, Japan. This discovery supports the idea that the ecological niche of the CL500-11 cluster is not limited to the hypolimnion of Crater Lake but also occurs in general aerobic deep freshwaters. In the present study, I quantitatively followed the vertical, horizontal, and seasonal distributions of the bacterium using catalyzed reporter deposition (CARD)-FISH. Additionally, 16S rRNA gene sequences, affiliated with the CL500-11 clusters that have been deposited in the Genbank/EMBL/DDBJ sequence database, were also examined, and by integrating the novel data with the currently available knowledge on the CL500-11 cluster, I provide new insights into the ecological characteristics of this bacterium.

Materials and Methods

Study site and sampling

Lake Biwa is a mesotrophic monomictic lake with a surface area of 674 km^2, a maximum depth of 104 m, and an estimated water retention time of 5.5 years. The lake has a permanently oxygenated hypolimnion, where the annual minimum dissolved oxygen concentration generally stays above 100 µM (Kim et al., 2006). Spatiotemporal measurements of dissolved and particulate C, N, and P in the lake are available in Kim et al. (2006). I collected water samples from pelagic stations in Lake Biwa (Fig. 2-1) using a 5-L Niskin-X bottle (General Oceanics, Miami, FL), and determined water temperature using a CTD profiler (SBE-911plus, Sea Bird Electronics, Sealogger, WA). Monthly samples were collected from Sta. 6 (water depth = ca. 73 m) from December 2009 to September 2011 (Fig. 2-1). Longitudinal samples were collected from Stations 3 through

7 in September 2011 (Fig. 2-1). The sampling depths were 5 m (epilimnetic water) and 3 to 6 depths between 35 m and 1 m above the bottom surface in 15-m intervals. For the first 3 months of the monitoring, I collected water samples from depths of 5 m, 30 m, 50 m, and the bottom (December 2009) or from the depths of 5 m, 25 m, 50 m, and the bottom (January and February 2010). Immediately after collection, water samples were fixed with glutaraldehyde (1% final concentration) for the enumeration of bacteria and with newly prepared buffered paraformaldehyde (pH 7.4, 1% final concentration) for use in CARD-FISH. All fixed samples were kept under cool conditions until further processing.

CARD-FISH

To detect the CL500-11 cluster in Crater Lake, Urbach et al. (2001) designed an oligonucleotide probe “CLGNS-584” for hybridization to membrane-blotted environmental rRNA. I used this same specific sequence to construct an HRP-labeled oligonucleotide probe (5’-GCCGACTTGCCCAACCTC-3’) (Thermo Fisher Scientific, MA). However, the CLGNS-584 probe alone did not produce sufficient signal intensity to clearly distinguish positive cells from the background. This is presumably because the target site of CLGNS-584 (*Escherichia coli* position 585-602) is located in the helix 22 of the 16S rRNA structure, where accessibility to the probe is low (Behrens et al. 2003). To improve the accessibility of CLGNS-584, I designed a helper oligonucleotide, which is an unlabeled oligonucleotide that binds at a location adjacent to the target site of the labeled probe and presumably enhances probe accessibility to the site by opening the secondary structure of the rRNA (Fuchs et al., 2000). The designed helper oligonucleotide, CLGNS-567h (5’ - CTACACGCCCTTTACGCC - 3’) targeted *E. coli* positions 567–584. According to the Probe Match tool of the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu), all 16S rRNA sequences targeted by CLGNS-584 had a completely complementary sequence to CLGNS-567h.

Total bacterial abundance was determined by the 4',6-diamidino-2-phenylindole (DAPI) method (Porter and Feig 1980), with at least 900 bacterial cells enumerated in each sample. The CARD-FISH procedure was performed as described by Pernthaler et

al. (2004), with some modifications. Briefly, within 6 h of fixation, 3 mL of the samples were filtered through a 0.2-μm polycarbonate filter. The collected cells were then coated with 0.1% (w/v) low-gelling-point agarose and permeabilized with lysozyme solution [0.05 M EDTA, 0.1 M Tris-HCl pH 8.0, 10 mg mL^{-1} lysozyme (MP Biomedicals, Santa Ana , CA)] for 40 min at 37°C. The filter was cut into 6 pieces and soaked with 300 μL of hybridization buffer [900 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.02% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) blocking reagent, 10% (w/v) dextran sulfate, 20% (v/v) formamide] containing 0.5 μg mL^{-1} of probe CLGNS-584 and 0.1 μg mL^{-1} of helper CLGNS-567h. The optimum formamide concentration was determined by testing a series of concentrations (0–50% at 10% intervals) to produce maximum signal intensity. The hybridization reaction was conducted in a 24-well microplate overnight at 46°C with mild agitation (10 rpm). After hybridization, the filter pieces were washed with 1× PBS twice for 10 min at 46°C. Stringent washing was omitted, as Wendeberg (2010) reported that it did not make a significant difference to CARD-FISH results, as CARD-FISH works with lower concentrations of the probe than does FISH using fluorochrome-labeled probes. I also tested the effect of the washing step and found that it did not affect the results. Fluorescein-labeled tyramide signal amplification was performed using the TSA Fluorescein System (NEL701001KT, Perkin Elmer, Waltham, MA). The filter pieces were then soaked in 30 μL of amplification mixture [1× amplification diluent:40% (w/v) dextran sulfate:fluorescein-tyramide reagent = 25:25:1] in a 24-well microplate and incubated in the dark for 45 min at 46 °C before being mounted on a glass slide with an anti-fading reagent [Citifluor (Citifluor, UK):Vectashield (Vector Laboratories, Burlingame, CA) = 4:1] containing 1 μg mL^{-1} of DAPI. For each sample, at least 1000 DAPI-positive cells and the corresponding FISH-positive cells were enumerated with UV and blue excitation, respectively, under an epifluorescence microscope. The CLGNS-584 positive cells were easily identified, because they showed bright fluorescence and a distinctive crescent shape. Negative control with the nonsense probe NON338 (Wallner et al., 1993) demonstrated that no false positive or autofluorescent objects were confused with the enumerated CLGNS-584 positive cells.

Cloning and phylogenetic analyses

To identify partial 16S rRNA gene sequences of CL500-11 bacteria, I performed a clone library analysis using a sample collected at a depth of 65 m at Sta. 6 (Fig. 2-1) in December 2010. Twenty-five milliliters of non-fixed sample were filtered through a 0.2-µm polycarbonate filter. DNA was extracted from the seston collected on the filter using cetyltrimethylammonium bromide, chloroform/isoamyl alcohol (24:1) and phenol/chloroform/isoamyl alcohol (25:24:1) according to Wilson (2001). The extracted DNA was resuspended in 40 µL of TE buffer. I used the universal primers 341f (Muyzer et al., 1993) and 907r (Muyzer et al., 1995) to amplify the eubacterial 16S rRNA gene. PCR amplification was performed in a 25 µl reaction mixture with a Blend Taq PCR kit (Toyobo, Japan) using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA). The PCR was performed under the following conditions: after 9 min preincubation at 94°C, 29 cycles at 94°C for 1 min, followed by annealing temperature for 1 min and 72°C for 3 min. In the first 19 cycles, the annealing temperature was reduced by 1°C after every two cycles from 64°C in the first cycle to 55°C in the nineteenth. During the last 10 cycles, the annealing temperature was 55°C, followed by a final extension at 72°C for 9 min. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, the Netherlands) and cloned using a pT7 Blue Perfectly Blunt Cloning Kit (Novagen, Madison, WI) according to the manufacturer's instructions. I picked 100 positive colonies and amplified the inserted DNA fragments with the T7 and U19 primers. The amplified fragments were verified by 2% agarose gel electrophoresis, and the DNA from the colonies, with an insert of the correct size, was sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) and an ABI3100-Avant Capillary Auto Sequencer.

The acquired partial 16S rRNA gene sequence of CL500-11 and the corresponding part (341–926 in *E. coli* numbering) of the related reference sequences retrieved by BLAST search (Altschul et al., 1997) were examined for phylogenetic analysis. The sequences were aligned using CLUSTALW and a neighbor-joining phylogenetic tree was then constructed using MEGA version 5.05 software (Tamura et

al., 2011). Only sequences covering >95% of the target segment (341–926 in *E. coli* numbering) were analyzed and included in the tree. Based on the constructed tree, related partial sequences covering <95% of the target segment were retrieved manually using BLAST search and the SeqMatch tool of the RDP. Sequences showing >98% similarity with the CL500-11 original full sequences (Accession number: AF316759) (Urbach et al., 2001) were considered to be affiliated with the CL500-11 cluster, and the resulting information was summarized.

Results

Temperature profile and bacterial abundance

The obtained temperature profile indicated that Lake Biwa was vertically mixed from January to March and thermally stratified from April to December (Fig. 2-2A). The water temperature ranged between 7.2°C and 30.2°C in the epilimnion (5 m) and was <10°C in the hypolimnion. In February 2010 and February 2011, differences in temperature between bottom and surface waters were <0.1°C, indicating that the lake was holomictic during the mixing periods of those 2 years.

During the stratified period, total bacterial abundance varied from 1.7×10^6 to 10.9×10^6 cells mL^{-1} in the epilimnion and 0.7×10^6 to 1.8×10^6 cells mL^{-1} in the hypolimnion. Exceptionally, 2.2×10^6 cell mL^{-1} was recorded from the bottom of the lake in July 2011. During the mixing period, bacterial abundance ranged from 1.3×10^6 to 3.0×10^6 cells mL^{-1} (Fig. 2-2B).

Spatiotemporal distribution of CL500-11 bacteria

CARD-FISH performed in conjunction with the helper oligonucleotide technique resulted in high signal intensity of positive cells; the CL500-11 cells thus detected exhibited curved-rod morphology with 1–2 µm length and 0.2–0.3 µm width (Fig. 2-3). The monthly monitoring revealed an annual cyclic vertical distribution of CL500-11 (Figs. 2-4A and B). The percentage of CL500-11 to DAPI-stained cells at 50 m depth during the stratified period in 2010 increased from below the detection limit in April to 12.3% (1.4×10^5 cells mL^{-1} in abundance) in December. Similar trends were found at 65 m and in

the bottom water, with percentages of 12.4% and 12.6% (1.1×10^5 and 1.3×10^5 cell mL^{-1}) in December 2010, respectively. At a depth of 35 m, the percentage also started to increase in April, reaching 8.8% (1.1×10^5 cell ml^{-1}) in September. Thereafter it decreased, presumably due to the collapse of the thermocline (Fig. 2-2A). In contrast, CL500-11 cells were generally undetectable in the epilimnion (5 m) during the stratified period in 2010 (Figs. 2-4A and B). Subsequent holomixis, however, distributed the CL500-11 cells across the water layers. The percentage of CL500-11 cells to DAPI-stained cells varied from 2.1% to 4.9% (0.4×10^5 to 1.2×10^5 cells mL^{-1}) at the studied depths in January 2011 and then decreased to 0.0–0.8% (0.0×10^5 to 0.1×10^5 cells mL^{-1}) in April 2011 (Figs. 2-4A and B).

The longitudinal distribution of the percentage of CL500-11 bacteria in relation to DAPI-stained cells in the hypolimnion (sampling depth, 35–103 m) ranged between 10.0% and 16.5% (1.0×10^5 to 1.9×10^5 cell ml^{-1}) in September 2011 (Figs. 2-4C and D). The percentage of CL500-11 bacteria in the epilimnion (sampling depth, 5 m) was below the detection limit at all stations.

Comparative analysis of 16S rRNA gene sequences

I obtained 83 sequences with clone library analysis. Eleven sequences were identical and showed 99.3% similarity to the original CL500-11 sequence (Accession number: AF316759) (Urbach et al., 2001). The acquired partial 16S rRNA gene sequence of the CL500-11 cluster, LB65D-54, was deposited in the DNA Data Bank of Japan (accession number: AB686531).

A phylogenetic tree indicated that the CL500-11 cluster is a member of the class *Anaerolineae* of the phylum *Chloroflexi* (Fig. 2-5). Pairwise similarities among the CL500-11 sequences exceeded 97.3% in all combinations. Clone 52-3-31, which was the closest clone located outside the CL500-11 cluster, showed 92.5% similarity to the clone LB65D-54 (Fig. 2-5). This sequence and four other sequences originating from deep oceans formed a distinct cluster ("deep-ocean cluster" in Fig. 2-5). Other *Anaerolineae* clones were derived from a variety of environments, including sediment, soil, sludge, symbiotic systems, and surface planktonic habitats (Fig. 2-5). Note that the analysis was

conducted using a 16S rRNA partial sequence (341–926 in *E. coli* numbering) and that the resulting tree was almost consistent with a tree of full-length analysis (Yamada and Sekiguchi, 2009).

Including partial sequences that were not assigned in the tree, I found 24 sequences affiliated with the CL500-11 cluster (Table 2-1). The sequences originated exclusively from freshwater environments. Four clones were reported from the hypolimnetic water of Crater Lake during the summer stratified period (Urbach et al., 2001). Four additional clones were retrieved from hypolimnetic samples collected from three deep subalpine lakes during their stratified periods, whereas six clones were collected from holomictic water samples (Humbert et al. 2009). In Yellowstone Lake, two studies individually investigated the bacterial community in the bottom waters near hydrothermal vents and found CL500-11 sequences (Clingenpeel et al., 2011; Yang et al., 2011). The other CL500-11 bacteria clones were reported from metalimnion (Van den Wyngaert et al., 2011), hypolimnion (Han et al., unpublished sequence), and holomictic (Allgaier and Grossart, 2006b) waters sampled from deep oxic lakes, except for two clones obtained from the tap water of a Norwegian hospital (Rudi et al., 2009).

Discussion

Ecological niche of the CL500-11 cluster

Using FISH counts, the present study first quantitatively followed annual succession in the CL500-11 population. The data indicate that CL500-11 bacteria are adapted to the stratified hypolimnion of Lake Biwa. Consistently, Urbach et al., (2007) have also reported high contributions of CL500-11 in the bacterial community of the oxygenated hypolimnion of Crater Lake during three consecutive summers. Moreover, almost all previously reported CL500-11 sequences were obtained from cells derived from oligo- to mesotrophic and holomictic lakes that had year-round oxygenated hypolimnion waters, with none derived from epilimnetic water during the stratification period (Table 2-1). Taken together, these findings suggest that CL500-11 bacteria may be generally distributed in the oxygenated hypolimnion of freshwater lakes. However, it is still

unclear whether CL500-11 forms a dominant cluster in lakes other than Crater Lake and Lake Biwa, as these bacteria have not yet been quantitatively evaluated in other lakes. It should be noted that extensive clone library analyses of the bacterial community of the oxygenated hypolimnion of Lake Baikal did not detect the presence of the CL500-11 sequence (Glöckner et al., 2000; Bel'kova et al., 2003). This suggests the existence of unknown environmental factors crucial for CL500-11 survival, although an oxygenated hypolimnion may be one of the necessary conditions. Ultraoligotrophy and growth limitation by N and trace metals, both of which are traits of Crater Lake water (Urbach et al., 2001, 2007), are not likely factors influencing CL500-11 occurrence because Lake Biwa is a mesotrophic, P-limited lake (Kim et al., 2006). Dissolved oxygen concentration in the hypolimnion is also unlikely to be a key factor, as it decreases by less than 150 μM in the late stratification period in Lake Biwa (Kim et al., 2006) and is generally >290 μM throughout the year in Crater Lake (McManus et al., 1996). As CL500-11 dominance has been reported only from these two lakes, the available data are not sufficient to identify the factors that potentially trigger the dominance of the bacteria. Quantitative information and comparative analyses of CL500-11 occurrences in lakes worldwide are needed in future studies.

Ecological diversification of CL500-11 within the phylum *Chloroflexi*

To date, only two phylogenetic groups of *Chloroflexi* are known to be predominant in aerobic planktonic habitats: the freshwater CL500-11 cluster and the marine SAR202 cluster (Giovannoni et al., 1996; Morris et al., 2004). The SAR202 cluster belongs to "subphylum IV" of the *Chloroflexi* phylum and is phylogenetically distant from the CL500-11 cluster in "subphylum I" (Yamada and Sekiguchi, 2009). Nonetheless, interestingly, the SAR202 cluster also dominates in deep-water environments, comprising 1.5 to 15% of all prokaryotic cells in deep layers of both the Atlantic and Pacific Oceans (Morris et al., 2004; Varela et al., 2008; Schattenhofer et al., 2009). It is also remarkable that the CL500-9 cluster, which is a subgroup of SAR202, was found in the bacterial community of the hypolimnion of Crater Lake, albeit not in high abundance (Urbach et al., 2001). Currently, very little is known about the ecophysiology of SAR202

bacteria. Varela et al. (2008) demonstrated that SAR202 bacteria preferably utilize L-aspartic acid (Asp) over D-Asp, although the percentages of bacteria taking up L-Asp decrease with depth. This result suggests that SAR202 may efficiently uptake available substrates in deep marine environments. Further studies of SAR202 bacteria are needed to elucidate its ecology in oxygenated deep-water environments, and this information may be applicable to future studies of CL500-11 bacteria.

It is also noteworthy that the CL500-11 cluster is phylogenetically grouped with a cluster derived exclusively from marine oxygenated deep waters ("deep-ocean cluster" in Fig. 2-5). The characteristics of the deep-ocean cluster are currently completely unknown because all of the reported sequences isolated from these waters have been documented as minor eccentric groups found in total bacterial community analyses (Pham et al., 2008; Galand et al., 2010; Redmond and Valentine, 2011; Swan et al., 2011). For instance, in a study of the deep Arctic Ocean, only 5% of the bacterial pyrosequencing reads belonged to phylum Chloroflexi, and the contribution of the deep-ocean cluster within the phylum was small (Galand et al., 2010). In a fosmid-based metagenomic analysis of the bacterial community at depths of 500 m and 770 m at the North Pacific Time-series Station ALOHA, sequences affiliated with the deep-ocean cluster accounted for less than 1% of the clones collected from both depths (Pham et al., 2008). Thus, it is likely that the deep-ocean cluster accounts for only a minor fraction of the deep marine bacterial community. Nonetheless, the striking similarities in habitats of the two sister clusters implies that they may have common ecophysiological traits. More ecological information on the deep ocean cluster should be collected for comparison with the CL500-11 cluster.

Clearly, it may not be appropriate to conclude the physiology of the CL500-11 cluster from that of its phylogenetic relatives. To date, only 6 species have been isolated and characterized in the class *Anaerolineae*, (Fig. 2-5), all of which show anaerobic growth, multicellular filamentous morphology, and mesophilic or moderately thermophilic preferences (Yamada and Sekiguchi, 2009). CL500-11 bacteria lack these characteristics. Additionally, the class contains environmental sequences derived from a

broad range of habitats (Fig. 2-5). The class also includes sequences from the surface planktonic habitat (Fig. 2-5), although individuals are less abundant in this habitat (Shaw et al., 2008; Van den Wyngaert et al., 2011). Thus, the class *Anaerolineae* contains a variety of bacteria, and its common characteristics are still unknown.

Ecological roles of the CL500-11 cluster

The longitudinal monitoring demonstrated that CL500-11 bacteria comprised more than 10% of the total bacterial population in the hypolimnion of Lake Biwa during the summer-stratified period (Figs. 2-4C and D). CL500-11 bacteria likely account for an even larger proportion of the community in terms of biomass because of their relatively large cell size (Fig. 2-3). This substantial abundance suggests that CL500-11 bacteria play a potentially important role in pelagic biogeochemical cycling in the lake. It is conceivable that CL500-11 bacteria utilize some dissolved substances for their growth, because almost all of the CL500-11 bacteria in this study were found to be free-living, and a subset were undergoing division at the time of analysis (arrows in Fig. 2-3B). Following a carbon stable isotope ratio analysis of the spatiotemporal dynamics of dissolved organic carbon (DOC) in Lake Biwa, Maki et al. (2010) suggested that a fraction of the DOC derived from surface photosynthetic production escapes from rapid degradation and accumulates in the epilimnion (semi-labile DOC), before being transferred to the hypolimnion by winter mixing and subsequently degraded over months during the stratified period. Thus, it may be that CL500-11 bacteria play a pivotal role in the recycling of the semi-labile fraction of DOC in the hypolimnion.

To clarify the substrate availability of CL500-11 bacteria, cultivation of an isolated strain will be crucial. However, a thorough effort by Page et al. (2004) to isolate previously uncultivated bacterial strains from Crater Lake using a high-throughput cultivation method (Connon et al., 2002), was unsuccessful in isolating CL500-11. This difficulty in isolation evokes another possibility regarding CL500-11 ecophysiology: mutualistic growth with other organisms. To support this, Urbach et al. (2007) have previously suggested that the dominance of CL500-11 in the hypolimnion in Crater Lake is related to the occurrence of marine Group I *Thaumarchaeota*, another predominant

phylotype in the hypolimnion. Callieri et al. (2009) also reported that the proportion of *Thaumarchaeota* to the total prokaryotic community in a deep oxic subalpine lake increased along with depth, although no information has been provided on the hypolimnetic eubacterial composition of the lake. These results imply that it would be worthwhile to investigate archaeal distribution and its relationship to CL500-11 succession in Lake Biwa.

Conclusions

In this study, CARD-FISH revealed a seasonal dominance of CL500-11 bacteria in the oxygenated hypolimnion of Lake Biwa, suggesting that CL500-11 is likely an important component of pelagic biogeochemical cycling in the lake. A search of previously deposited CL500-11 sequences in clone libraries suggested that the bacteria might be ubiquitously distributed in oxygenated hypolimnion waters. Therefore, exploration of the bacterial community composition in oxic lakes worldwide and isolation of these bacteria would further elucidate CL500-11 ecophysiology.

Tables and Figures

Table 2-1

Details of the lakes from which the 24 database clones of the CL500-11 cluster originated.

Sampling site	Country	Mean (max.) depth (m)	Area (km^2)	Trophic status	Stratified period	Year-round oxygenated hypolimnion	Sampling depth (m)	Sampling month [a]	No. of clones	Accession number	Reference
Lake Biwa	Japan	41 (104)	670	Mesotrophic	Apr. - Dec.	yes	65	Dec. (s)	1	AB686531	This study
Crater Lake	USA	350 (594)	53	Oligotrophic	May - Nov.	yes	120	Aug. (s)	1	AF316758	Urbach et al., 2001
							500	Aug. (s)	3	AF316759-AF316761	Urbach et al., 2001
Lake Annecy	France	42 (65)	28	Oligotrophic	May - Nov.	yes	2	Jan. (m)	1	AJ965812	Humbert et al., 2009
							50	Jan. (m)	1	AJ965898	Humbert et al., 2009
							50	Aug. (s)	2	AJ965858, AJ965870	Humbert et al., 2009
Lake Bourget	France	85 (145)	45	Mesotrophic	Apr. - Nov.	yes	50	Apr. (s)	1	AJ966070	Humbert et al., 2009
Lake Geneva	France /Switzerland	154 (310)	580	Mesotrophic	Apr. - Nov.	yes	2	Jan. (m)	4	AJ966118,AJ966120, AJ966121,AJ966124	Humbert et al., 2009
							50	Apr. (s)	1	AJ966228	Humbert et al., 2009
Lake Zurich	Switzerland	49 (143)	89	Mesotrophic	May - Nov.	yes	15	Oct. (s)	2	FN668367, FN668368	Van den Wyngaert et al., 2011
Lake Breiter Luzin	Germany	22 (59)	3.6	Mesotrophic	Jun. - Oct.	yes	0-10	Nov. (m)	1	DQ501287	Allgaier and Grossart, 2006b
Lake Soyang	South Korea	42 (110)	44	Mesotrophic	May - Dec.	yes	80	unknown	1	AF107533	Han et al. (unpublished)
Yellowstone Lake	USA	42 (120)	53	Oligotrophic	May - Nov.	yes	21.7 [b]	Jul (s)	1	HM446117	Yang et al., 2011
							52.4 [b]	Jul (s)	1	HM446145	Yang et al., 2012
							unknown [c]	unknown	1	HM856384	Clingenpeel et al., 2011
Tap water	Norway	n/a	n/a	n/a	n/a	n/a	n/a	n/a	2	GQ165424, GQ165425	Rudi et al., 2009

a. (s): stratified period; (m): mixing period.

b. Hydrothermal vent water.

c. Samples were obtained from the near-surface photic zone or hydrothermal vents.

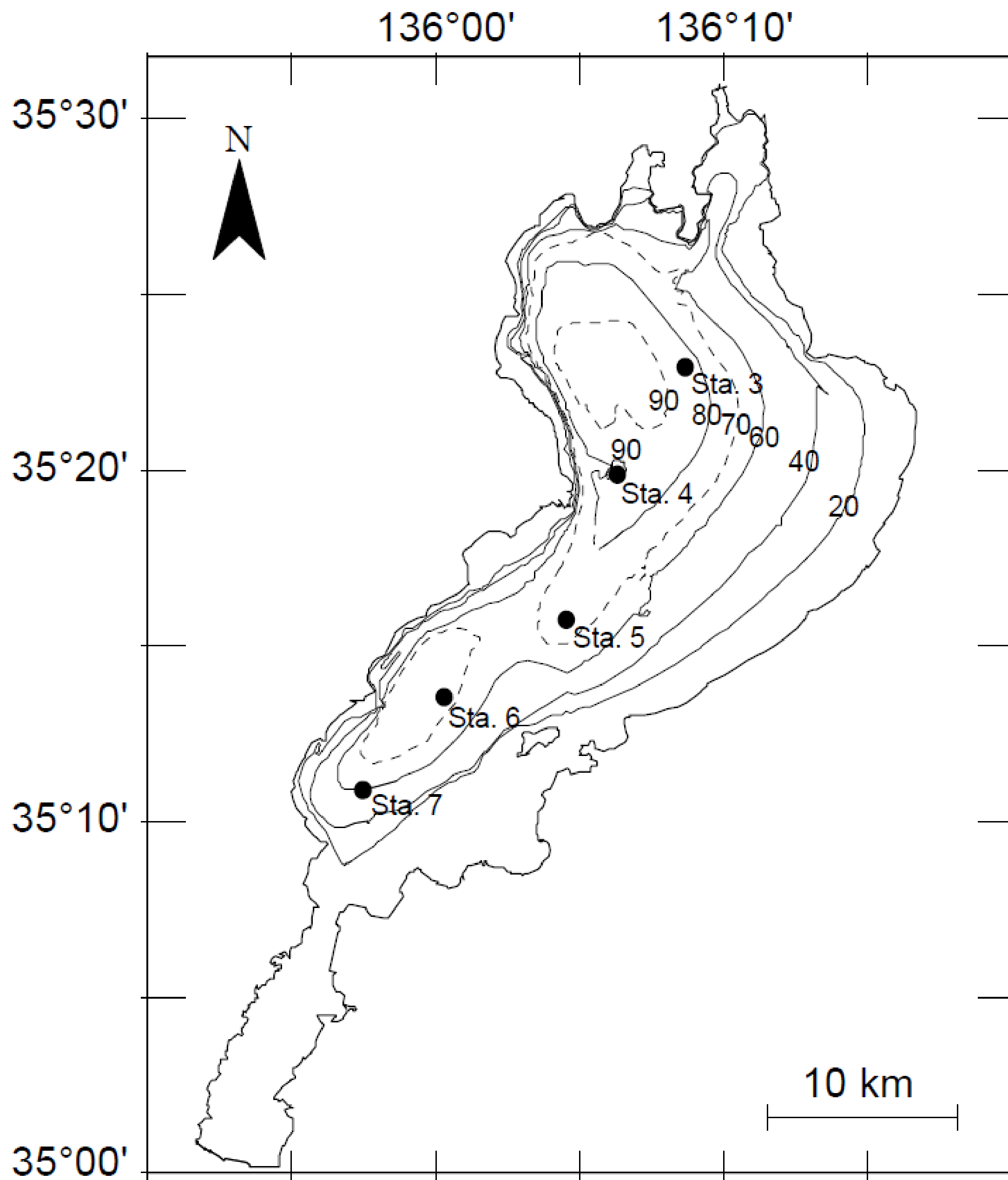


Figure 2-1

Lake Biwa sampling stations. Depths are shown in meters.

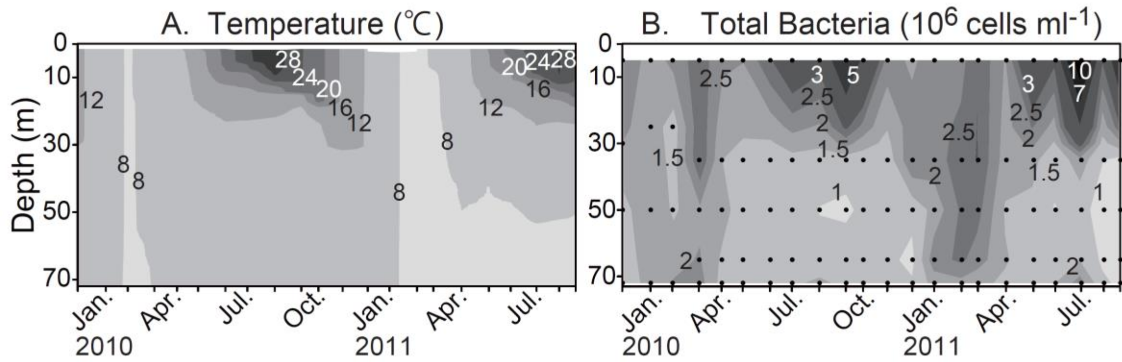


Figure 2-2

(A) Water temperature and (B) abundances of total bacterial (DAPI-stained) cells at the monthly monitoring station (Sta. 6) during the study period. Dots indicate sampling points.

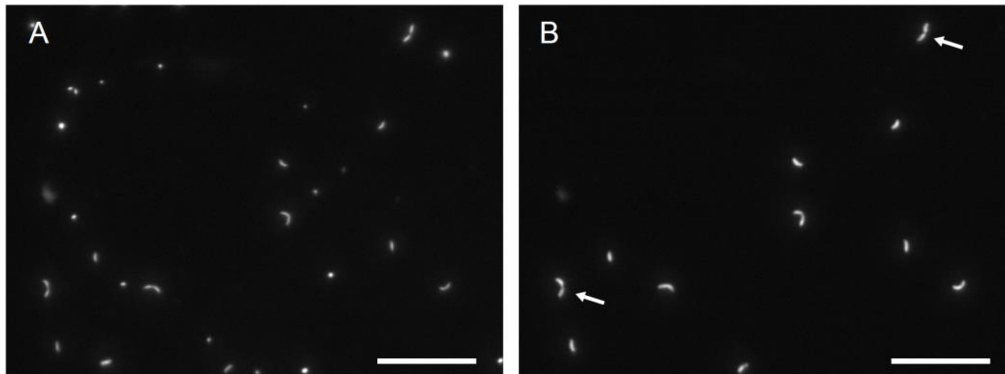


Figure 2-3

CARD-FISH images of CL500-11 cells. An identical microscopic field of (A) DAPI-stained cells and (B) CL500-11 bacteria stained by CARD-FISH with a combination of probe CLGNS-584 and helper CLGNS-567h. The sample was taken at Sta. 4 at a depth of 50 m in September 2011. Arrows indicate dividing cells. Images were acquired using a CCD camera (Hamamatsu ORCA-ER, Japan) attached to a microscope with a fixed exposure time (22.0 ms). The scale indicates 10 µm.

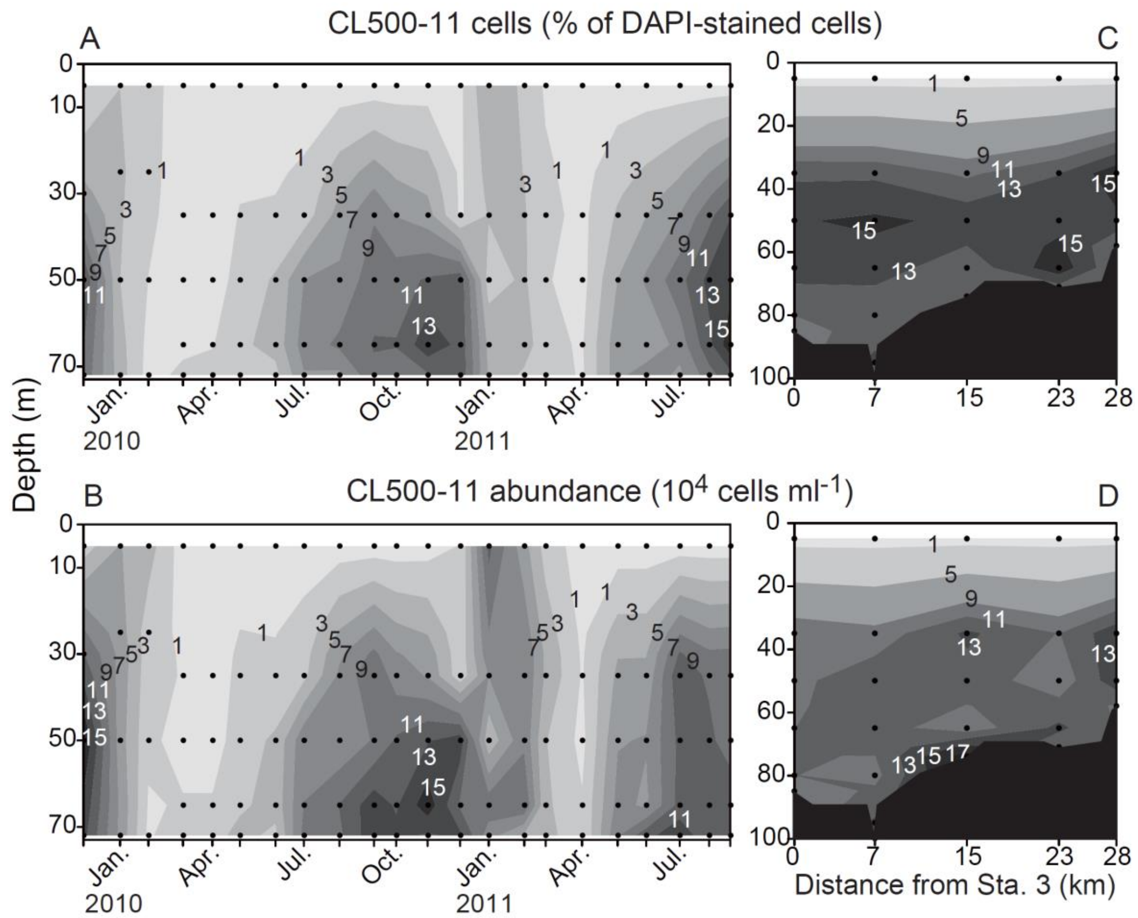


Figure 2-4

The percentage of CL500-11 to DAPI-stained cells and CL500-11 abundance (A and B, respectively), determined by monthly monitoring at Sta. 6 and by longitudinal monitoring in September 2011 (C and D, respectively). Dots indicate sampling points.

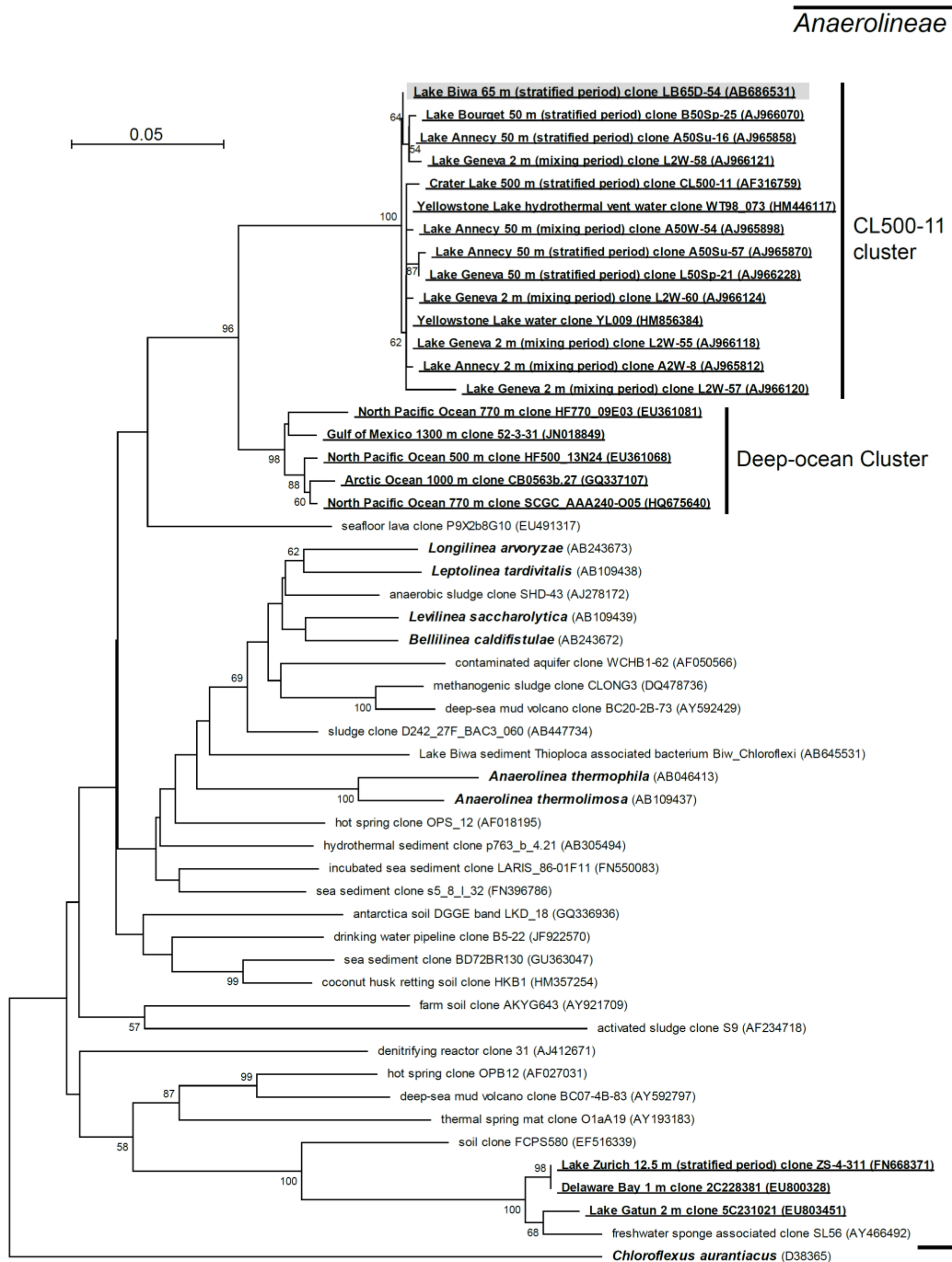


Figure 2-5

Phylogenetic tree of the CL500-11 cluster and representatives of the class *Anaerolineae*, derived from partial sequences of 16S rRNA gene (*E. coli* position 341-926). The sequence deposited in the present study (LB65D-54) is shaded grey. Cultivated strains are shown in bold, whereas uncultivated gene clones are labeled with the environments from which they originated. Planktonic gene clones are underlined. Nodes supported by bootstrap value of >50% are indicated. The scale bar represents five nucleotide substitutions per 100 nucleotides.

Chapter 3: Comprehensive investigation of hypolimnion-specific bacterioplankton lineages in Lake Biwa

Introduction

With the development of cultivation-independent molecular tools, the understanding of the ecology of freshwater bacterioplankton has experienced unprecedented growth. In particular, constructing comprehensive and consistent knowledge of globally distributed phylogenetic groups (Glöckner et al., 2000; Zwart et al., 2002) has facilitated the identification of the vast majority of uncultured taxa. In the latest taxonomic framework, a fine taxonomic unit named tribe, which contains >97% sequence identity over the full length of the 16S rRNA gene, was proposed (Newton et al., 2011). Following this nomenclature, studies have been attempting to reveal the ecophysiology of individual uncultivated tribes to understand their roles in biogeochemical cycling and microbial food webs (Eckert et al., 2012; Eiler et al., 2012; Salcher et al., 2013; Tada and Grossart, 2014). Such a fine phylogenetic resolution is necessary, because ecophysiological characteristics are often different even among closely related tribes, as was found among members of acI (Garcia et al., 2012; Ghylin et al., 2014), *Polynucleobacter* (Hahn et al., 2012; Watanabe et al., 2012), and *Limnohabitans* (Kasalický et al., 2013; Šimek et al., 2014).

Despite the ecological importance of oxygenated hypolimnion (Chapter 1), available knowledge on freshwater bacterioplankton largely focuses on the epilimnetic community (Newton et al., 2011). The discovery of a hypolimnion-specific lineage (i.e., CL500-11) in Lake Biwa (Chapter 2) suggests that there can be hypolimnion-specific lineages other than CL500-11. While there is growing evidence for bacterioplankton groups that exclusively occur in the oxygenated hypolimnion (Urbach et al., 2001; Pollet et al., 2011; Callieri et al., 2015), only few studies have conducted comprehensive community analysis (i.e., cloning-sequencing of the 16S rRNA gene) in the realm

(Glöckner et al., 2000; Urbach et al., 2001; Bel'kova et al., 2003; Humbert et al., 2009). The available knowledge is currently insufficient to reach a firm understanding of bacterioplankton inhabiting the oxygenated hypolimnion.

The present study aimed to acquire an overview of the bacterioplankton community composition in an oxygenated hypolimnion using high-throughput sequencing of the 16S rRNA gene amplicon. Samples were spatiotemporally taken in a mesotrophic, holomictic (monomictic) deep lake with a fully oxygenated hypolimnion (Lake Biwa, Japan), and the sequences were analyzed with a fine phylogenetic resolution (i.e., distinguishing <97% identical sequences). This allowed us to demonstrate that the bacterioplankton community in the hypolimnion is significantly different from that in the epilimnion and to characterize groups showing hypolimnion-specific distribution patterns, which have eluded researchers due to their absence in the epilimnion.

Materials and Methods

Sampling procedure

Monthly sampling was conducted from March 2010 to June 2011 (except for February 2011) at a pelagic site of Lake Biwa (35°12′58″N 135°59′55″E; depth ca. 73 m; Fig. 3-1). In each month, the samples were collected from 5 m, 50 m, and 72 m (i.e., 1 m above the bottom), using a 5 L Niskin-X bottle (General Oceanics, FL). Water samples were refrigerated until further processing. The water temperature and total prokaryotic abundance were determined as described previously (Okazaki et al., 2013).

DNA extraction, amplification, and pyrosequencing

For DNA extraction, prokaryotic cells in a 25 mL water sample were collected on a 0.2 μm polycarbonate filter. Samples were maintained at −20°C until DNA was extracted by the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. For amplicon sequencing, the V4 and V5 regions of the 16S rRNA gene were amplified with modified 530F and 907R primers, which can target a broad range of bacteria and archaea (Nunoura et al., 2012). A two-step PCR was employed to efficiently obtain amplicons, including an eight-base-pair DNA tag (for post-

sequencing sample identification), and the 454 adaptors conjugated on both sides at the end. The primer sequences and PCR conditions used are provided in Tables 3-1 and 3-2, respectively. Each PCR step was performed in a 25 µL volume with the buffer system of Blend Taq Plus (TOYOBO, Osaka, Japan), followed by purification with the UltraClean PCR Clean-Up Kit (MoBio Laboratories, Carlsbad, CA, USA).

The final product from each sample was equimolarly pooled according to quantification by a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing was performed in the 1/8 regions of a sequencing reaction of a GS-FLX sequencer (Roche 454 Titanium) (Macrogen Japan Corp. Kyoto, Japan).

Operational Taxonomic Unit (OTU) creation and basic analysis

Sequence data were analyzed using the UPARSE pipeline (Edgar, 2013) by subsequently applying commands and scripts following the author's guideline. I used a fastq_maxee value of 1.0, a truncated length of 200 bp, and an OTU creation identity threshold of 97%. Thereafter, taxonomic assignment of individual OTU was performed by SINA 1.2.11 (Pruesse et al., 2012) referring SILVA 123 classification (Quast et al., 2013). Subsequently, non-prokaryotic OTUs (i.e., chloroplast, eukaryote, and unclassified domain) were removed.

I only used the V4 region (i.e., the truncated length of 200 bp) of the 16S rRNA gene in the analysis. If the V5 region was included (i.e., a truncated length of 350 bp), the number of total reads was considerably lower (< 40% of a 200 bp dataset), since longer reads are more prone to removal by the quality filtering step in the pipeline.

The alpha and beta diversity analysis were carried out by the phyloseq (McMurdie and Holmes, 2013) and the vegan (Oksanen et al., 2015) packages of the R software (http://www.R- project.org/), respectively.

Identification of the predominant OTUs

Predominant OTUs were further identified following the freshwater bacterioplankton taxonomic framework (Newton et al., 2011). Using NCBI BLAST+ tools 2.2.31 (Camacho

et al., 2009), each OTU was mapped against a reference fasta-formatted database (containing 11,587 partial and full 16S rRNA sequences) that was created by the ARB software (Ludwig, 2004) from the original data provided by Newton et al. (2011). If an OTU showed a >99% similarity to a reference sequence, the OTU was identified by following the phylogeny of the reference sequence in the original ARB data. I followed the finest naming structure available in the reference sequence, such as "tribe" (>97% identity over the full-length 16S rRNA sequence), "clade" (>95%), or "lineage" (85%–90%), as proposed in the literature (Newton et al., 2011). OTUs that failed to be named by this procedure were identified using the NCBI BLAST online search against the public sequence database (http://www.ncbi.nlm.nih.gov/). Each OTU was named following the name of the hit sequence that had the highest and >99% identity. If there were multiple candidates, the OTU was preferentially named after published sequences with the prefixes CL- (Urbach et al., 2001), LiUU- (Eiler and Bertilsson, 2004), and P-OTU- (Pollet et al., 2011). For members of the genus *Limnohabitans*, I followed the nomenclature proposed by Kasalický et al. (2013). For nitrifying bacteria, the more widely accepted nomenclature of genera was used. For cyanobacteria, identification was not carried out, since the 200 bp information is insufficient for taxonomic assignment.

Results and Discussion

The high-throughput sequencing produced 133,266 reads of the V4 region of the 16S rRNA gene amplicon from the 45 samples taken in 15 months at three different depths (5, 50, and 72 m) at a pelagic site of Lake Biwa (Fig. 3-1). The analysis generated 859 OTUs that are affiliated with bacteria and four OTUs that are affiliated with archaea. Nucleotide sequence data reported in this study are available in the Sequence Read Archive database under accession numbers DRX048052–DRX048095 (BioProject: PRJDB4503).

Analysis of the Shannon diversity index in each sample (Fig. 3-2A) indicated that the alpha diversity between the epilimnion and hypolimnion samples during the stratification period (April to December; Fig. 3-1) was not significantly different (p =

0.156; Wilcoxon test). This means that the phylogenetic richness and the evenness of the bacterioplankton community in both water layers were generally comparable. However, an analysis of the beta diversity between the samples by non-metric multidimensional scaling (NMDS) revealed that the community composition in the epilimnion and hypolimnion were different from each other (Fig. 3-2B). The samples from 50 and 72 m were plotted closely together and showed similar succession patterns, and the highest divergence from the epilimnetic samples occurred from October to December (Fig. 3-2B). In the epilimnion, samples taken from August to October showed the highest divergence, and the samples from the mixing period (January–March) were positioned between the plots from both layers (Fig. 3-2B). These results indicated that the thermocline separates the bacterioplankton community. In Lake Biwa, the strongly stratified mid-summer epilimnion is characterized as the nutrient deficient (Kim et al., 2006) and DOC-rich (Maki et al., 2010; Thottathil et al., 2013) clear water phase. Such a severe condition may have selected groups that were acclimatized to this environment and resulted in characteristic communities in the epilimnion from August to October (Fig. 3-2B). On the other hand, in the hypolimnion, an accumulation of semi-labile (Maki et al., 2010) or humic-like (Thottathil et al., 2013) refractory DOM throughout the stratification period has been suggested. The selection of the bacterioplankton that is capable of utilizing this less bioavailable DOM that accumulates in the hypolimnion may have resulted in the characteristic communities that were observed at the end of the stratified period (Fig. 3-2B).

At the phylum-level of phylogenetic resolution, *Bacteroidetes* and *Actinobacteria* were predominant in the epilimnion during stratification, accounting for 37.3%–59.5% and 8.9%–39.3% of the total amplicon reads, respectively (Fig. 3-3A). The *Chloroflexi* and *Planctomycetes* became relatively abundant in the hypolimnion during stratification, ranging from 1.3% to 31.5% and 3.4% to 23.1% respectively (Fig. 3-3A). It should be noted that these data potentially include some biases and should be considered with caution. First of all, the total prokaryotic abundance by season and depth varied more than six fold in this study (Fig. 3-1). Consequently, the proportion of amplicon reads

should not be directly related to the abundance. Furthermore, the value is potentially biased by the processes of DNA extraction and amplification (McCarthy et al., 2015; von Wintzingerode et al., 1997) and by uneven copy numbers of the 16S rRNA gene among target organisms (Farrelly et al., 1995). For example, according to the rrnDB database (Stoddard et al., 2015), the *Chitinophagaceae*, which includes members of bacI, one of the most represented Bacteroidetes in the present study, had as high as 3–6 copies of the rrn operon per chromosome, which may have resulted in the high read proportion of *Bacteroidetes* in the data (Fig. 3-3). Nevertheless, I still found clear patterns, i.e., the same taxa could be either highly represented (e.g., > 5% of the total reads) or nearly absent (e.g., < 0.5% of the total reads) among different samples. The pattern is particularly clear when I compare data between the epilimnion and hypolimnion during stratification, as shown in the beta diversity analysis (Fig. 3-2B).

To investigate the difference between the epilimnetic and hypolimnetic communities, data from each layer during the stratified period were pooled (Fig. 3-3B). Then the 30 predominant OTUs in each layer (a total of 49 OTUs because 11 OTUs were shared by both water layers) were identified. Consequently, 26 OTUs were identified by following the taxonomic framework by Newton et al. (2011), and the other 23 OTUs were named following the name of the BLAST hit sequence that had the highest and >99% identity.

The dominant members of the epilimnetic community were affiliated with previously known groups: the acI-B1, acI-C2, acI-A7, and Iluma-A1 tribes (*Actinobacteria*), the bacI-A1 tribe, the bac-II-A clade, and the bacV and bacI lineages (*Bacteroidetes*) (Newton et al., 2011), and the LimC cluster of *Limnohabitans* (*Betaproteobacteria*) (Kasalický et al., 2013) (Fig. 3-3B). Other well-studied tribes of LD12 (Salcher et al., 2011a; Heinrich et al., 2013), PnecB of the *Polynucleobacter* (Wu and Hahn, 2006a; Salcher et al., 2011b; Hahn et al., 2012), and LD28 (Salcher et al., 2015) were less abundant but ranked as one of the representative groups of the epilimnetic community (Fig. 3-3B). In the hypolimnetic community, while bacI-A1, acI-B1, acI-A7, Iluma-A1, and LimC showed high proportion, CL500-11 of the *Chloroflexi*

(Urbach et al., 2001) was the most represented (Fig. 3-3B). In addition, CL500-15, CL500-37, CL500-3 (*Planctomycetes*) (Urbach et al., 2001), LiUU-3-374 (*Acidobacteria*), and LiUU-3-330 (*Chlorobi*) (Eiler and Bertilsson, 2004) were highly represented in the hypolimnetic community (Fig. 3-3B).

I further inspected the vertical preferences of the individual groups based on their read percentage (Fig. 3-4) and patterns of appearance in each layer (Fig. 3-5). Members of the acI lineage were reported to consume the relatively bioavailable DOM, such as amino acids, polyamines, di- and oligopeptides, and carbohydrates (Garcia et al., 2012; Salcher et al., 2013; Ghylin et al., 2014). Other studies have proposed an "opportunistic" nature of *Bacteroidetes* (Eiler and Bertilsson, 2007; Zeder et al., 2009; Salcher, 2013) and *Limnohabitans* (Šimek et al., 2011, 2014; Salcher, 2013), which quickly respond to phytoplankton blooms by rapidly exploiting fresh photosynthetic products. The fact that bacI-A1, acI-A1, acI-A7, and LimC could sustain their population, even in the dark stratified hypolimnion (Figs. 3-4 and 3-5), suggests that they are not absolutely dependent on fresh and labile photosynthetic products. It is possible that they are passively transported from the epilimnion as particle-associated bacteria because high sinking fluxes of blooming phytoplankton (Kagami et al., 2006) and cyanobacteria (Takasu et al., 2015) have been reported in the lake. Because I only used the V4 region of the 16S rRNA gene for the analysis, I may not be able to detect the difference of the genotype in the same OTU between the epilimnion and hypolimnion. Nonetheless, the data demonstrated that the groups ubiquitous in the surface freshwater habitat could also be predominant in the deeper (> 50 m) aerobic layers.

The epilimnion-specific distribution patterns of LD12 and PnecB (Figs. 3-4 and 3-5) were in agreement with previously reported vertical profiles (Wu and Hahn, 2006b; Salcher et al., 2011a, 2011b). The present data demonstrated that several other groups (e.g., acI-C2 and bacII-A) also showed preferences to the epilimnion (Figs. 3-4 and 3-5). They presumably consume substrate that is only available in the euphotic layer (e.g., labile photosynthetic products from phytoplankton) or prefer the higher temperature in the epilimnion (Fig. 3-1). It is also possible that they possess a light-driven metabolic

pathway and prefer the euphotic surface water. The presence of rhodopsin genes among members of acI, *Bacteroidetes*, LD12, and *Polynucleobacter* has been suggested (Atamna-Ismaeel et al., 2008; Sharma et al., 2009; Martinez-Garcia et al., 2012; Ghylin et al., 2014).

The dominance of *Chloroflexi* CL500-11 bacterioplankton in the hypolimnion during stratification in the lake (Figs. 3-3B and 3-4) has been previously reported using fluorescent *in situ* hybridization (Okazaki et al., 2013). In this study, I further identified *Planctomycetes* CL500-15, CL500-37, and CL500-3 as highly represented hypolimnion-specific groups (Figs. 3-3B, 3-4, and 3-5). All the four groups (with the prefix "CL500-") were originally reported as representative bacterioplankton in the oxygenated hypolimnion (500 m deep) of the ultraoligotrophic Crater Lake (USA) during the stratified period (Urbach et al., 2001, 2007). This suggests that these groups are not endemic but are commonly distributed in the oxygenated hypolimnion of freshwater lakes.

CL500-15, a member of an uncultured class OM190, was highly represented in the hypolimnion, with as high as 11.1% of the total amplicon reads in August at 72 m (Fig. 3-4). To the best of my knowledge, this is the first study of such a high frequency of CL500-15 detection, although small numbers of clone library sequences were found in the oxygenated hypolimnion of Crater Lake (Urbach et al., 2001), Lake Annecy, and Lake Bourget (France) (Pollet et al., 2011) (referred to as OTU45 in the literature). CL500-37 and CL500-3 are affiliated with the *Phycisphaeraceae*, with an 85% identity in 611 bp of the original 16S rRNA gene partial sequence [note that CL500-37 was regarded as a member of the CL500-3 cluster in the studies in Crater Lake (Urbach et al., 2001, 2007)]. It is remarkable that neither were found in the oxygenated hypolimnion of Lake Annecy or Bourget (Pollet et al., 2011), while both were present in the winter mixing water of the deep oligotrophic Lake Stechlin (Germany) (Tada and Grossart, 2014) (referred to as OTU22 and OTU10 in the literature, respectively). In addition, four other *Planctomycetes* belonging to the *Planctomycetaceae* were also found as hypolimnion-specific groups: P-OTU1, P-OTU76, P-OTU31, and CL500-52 (Figs. 3-3B, 3-4, and 3-5).

In the oxygenated hypolimnion of Lake Annecy and Bourget, P-OTU1 and CL500-52 (referred to as OTU2 in the literature) were predominant members of *Planctomycetes*, while P-OTU76 and P-OTU31 were less represented (Pollet et al., 2011). Altogether, *Planctomycetes* may be a phylum that is generally distributed in the oxygenated hypolimnion with different predominating members at different habitats. Their high diversity (covering the three classes) across the phylum supports the idea that their unique physiological characteristics shared among the phylum might enable their successful dominance in the hypolimnion; for example, nucleoid compartmentalization and endocytosis-like protein uptake are thought to be common characteristics of the phylum (Fuerst and Sagulenko, 2011). I propose that members of *Planctomycetes* should receive more attention in future studies given their potentially important roles in deep freshwater ecosystems.

The occurrence of *Ca.* Nitrosoarchaeum of ammonia-oxidizing archaea (AOA) in the oxygenated hypolimnion (Figs. 3-3B, 3-4, and 3-5) has also been reported in Crater Lake (Urbach et al., 2001), Lake Redon (Spain) (Auguet et al., 2012), Lake Maggiore (Italy/Switzerland) (Coci et al., 2015), and Lake Superior (USA/Canada) (Mukherjee et al., 2016). In the previous studies, niche separation of AOA and ammonia-oxidizing bacteria (AOB) was suggested between lakes (Mukherjee et al., 2016) and depths (Coci et al., 2015) but is unlikely between seasons (Auguet et al., 2012). The data demonstrated that AOA only occurred at the later phase of the stratified period, while AOB (*Nitrosospira*) more continuously occurred in the hypolimnion (Figs. 3-4 and 3-5). Another group of nitrifiers, *Nitrospira*, were also represented in the hypolimnion during the stratification (Figs. 3-4 and 3-5), yet only sporadic reports are available on *Nitrospira* inhabiting the oxygenated water columns of freshwater lakes (Bel'kova et al., 2003; Tada and Grossart, 2014; Mukherjee et al., 2016). In Lake Biwa, nitrate accumulation in the hypolimnion throughout the stratified period with a constantly low ammonium concentration (order of nM) was reported (Kim et al., 2006; Thottathil et al., 2013). Although direct evidence is lacking, it is likely that the three nitrifiers were involved in nitrification in the water column of the oxygenated hypolimnion of the lake.

In the data, two members of uncommon phyla were also identified as hypolimnion-specific groups: LiUU-3-374 (*Acidobacteria*) and LiUU-9-330 (*Chlorobi*) (Figs. 3-4 and 3-5), both of which were originally described in Swedish lakes (Eiler and Bertilsson, 2004). Their closest relatives found in the public sequence database were almost exclusively retrieved from natural freshwater environments (data not shown). This and the fact that the samples were from a pelagic lake site (Fig. 3-1) together indicate that they are indigenous and were not transported from an allochthonous source. In the data, the LiUU-3-374 highly represented from the winter mixing period to the early stratified period (Fig. 3-4). Considering that the only isolated strain in the same family (*Holophagaceae*) is able to degrade methoxylated aromatic compounds (Liesack et al., 1994), it is plausible that LiUU-3-374 contribute to refractory DOM degradation in the lake ecosystem, as was proposed for members of deep sea Acidobacteria (Quaiser et al., 2008). LiUU-9-330 was affiliated with an uncultured class OPB56, which is a deeply branched lineage in the *Chlorobi* phylum (Hiras et al., 2015). Although their ecological characteristics remain unknown, it is likely that they are not strictly hypolimnion-specific because many closely related sequences in the public database were reported from the surface waters of mesotrophic (e.g., FN668200 by Van den Wyngaert et al., 2011) to even hypereutrophic (e.g., JN371709 by Li et al., 2012) lakes. In the data, LiUU-9-330 were not exclusively detected in the hypolimnion but also occurred in the mid-summer epilimnion (Fig. 3-4).

Conclusions

The present is the first comprehensive investigation of the bacterioplankton community composition in the oxygenated hypolimnion of a freshwater lake covering the whole stratification period. The analysis identified many phylogenetic groups that exclusively occurred in the hypolimnion, which were understudied by previous research that is targeting only the epilimnion. Future studies focusing on the ecophysiology of these individual hypolimnion specialists are crucial for further understanding the microbial ecology and biogeochemical cycling in the pelagic freshwater ecosystem.

Tables and Figures

Table 3-1

PCR primers used in the present study.

Name	Sequence (5' to 3')	Target*
First PCR		
Forward 530F-mix**		
Bac 530F	GTGCCAGCAGCCGCGG	Most of Bacteria and some Archaea
Bac2 530F	GTGCCAGCAGCWGCGG	Some OP11, OD1 and diverse bacterial groups
Bac3 530F	GTGCCAGCAGTCGCGG	Some OP11, OD1 and diverse bacterial groups
Arch 530F	GTGBCAGCCGCCGCGG	Most of Archaea
Reverse 907R-mix**		
Uni 907R	CCGYCAATTCMTTTRAGTTT	Most of Bacteria and Archaea
DeepAB 907R	CCGYCTATTCCTTTGAGTTT	Desulfurobacteriaceae, OD1 and diverse bacterial and archaeal groups
SAG-Del 907R	CCGYCAATTTCTTTRAGTTT	Some Deltaproteobacteria, SAGMEG and diverse bacterial and archaeal group
Second PCR***		
Adaptor A – tag – 530F	CCATCTCATCCCTGCGTGTCTCCGACTCAG NNNNNNNN GTGBCAGCMGYHGCGG	The forward end of the first PCR product
Adaptor B – 907R	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG CCGYCWATTYMTTTRAGTTT	The revearse end of the first PCR product

*Organisms targeted by each primer were available in Nunoura et al. (2012).

**For the first PCR, primers were mixed equimolarly for each of the forward and reverse set.

***For the second PCR, primers were designed to target all combinations of the first PCR product, conjugating sample-specific 8 bp tags (the underlined Ns) and the 454 adapters at both ends. PCR conditions are shown in the table on the right.

Table 3-2

PCR conditions used in the present study.

	PCR Conditions				PCR Mixture (25.0 μL)	
First PCR					10×Buffer	2.5 μL
	Preincubation	94 °C	4 min		dNTPs	2.5 μL
	Denaturation	94 °C	1 min	× 30	Taq solution	0.2 μL
	Annealing	64 → 55 °C*	1 min		Forward primer (5 μM)	2.0 μL
	Elongation	72 °C	2 min		Reverse primer (5 μM)	2.0 μL
	Final elongation	72 °C	4 min		Template	5.0 μL
					Water	10.8 μL
Second PCR					10×Buffer	2.5 μL
	Preincubation	94 °C	4 min		dNTPs	2.5 μL
	Denaturation	94 °C	1 min	× 5	Taq solution	0.2 μL
	Annealing	55 °C	1 min		Forward primer (5 μM)	1.0 μL
	Elongation	72 °C	2 min		Reverse primer (5 μM)	1.0 μL
	Final elongation	72 °C	4 min		Template	5.0 μL
					Water	12.8 μL

*In the first 19 cycles, the annealing temperature was reduced by 0.5°C after every cycle from 64°C to 55°C. During the last 10 cycles, the annealing temperature was 55°C.

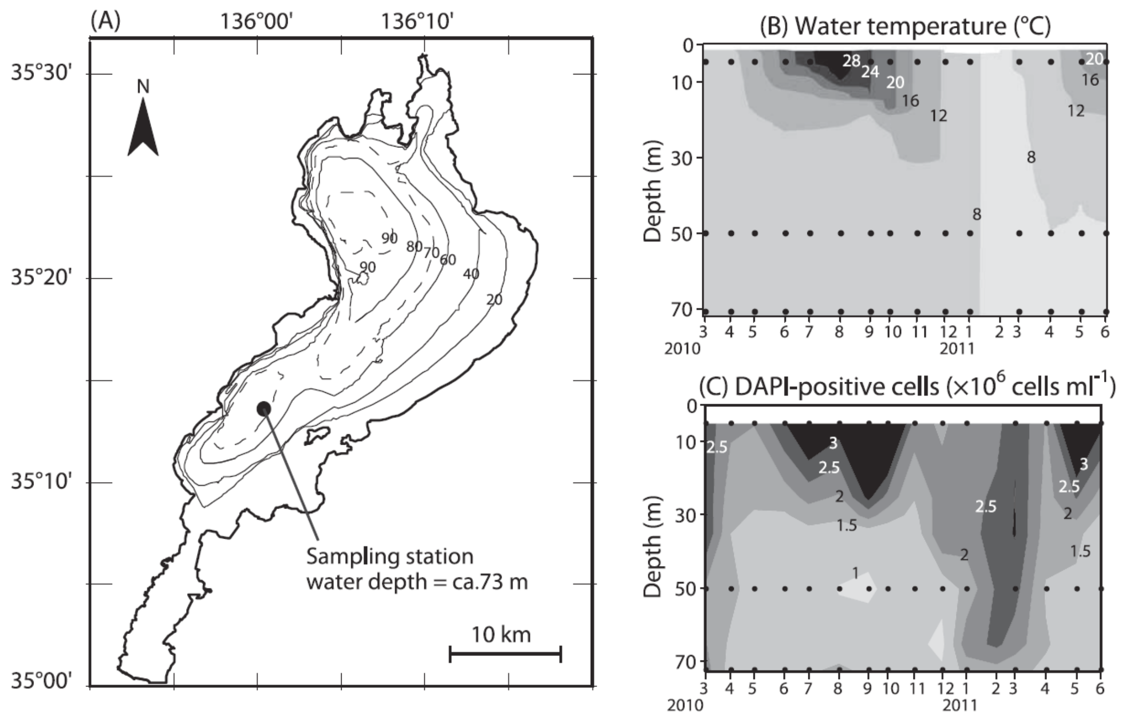


Figure 3-1

Basic information on the study site. (A) Location of the sampling site (Lake Biwa, Japan). (B) A spatiotemporal profile of water temperature determined by a CTD profiler. (C) A spatiotemporal profile of total prokaryotic abundance determined by enumeration of DAPI-positive cells. Dots indicate the depths and months from which the samples for the community analysis were taken.

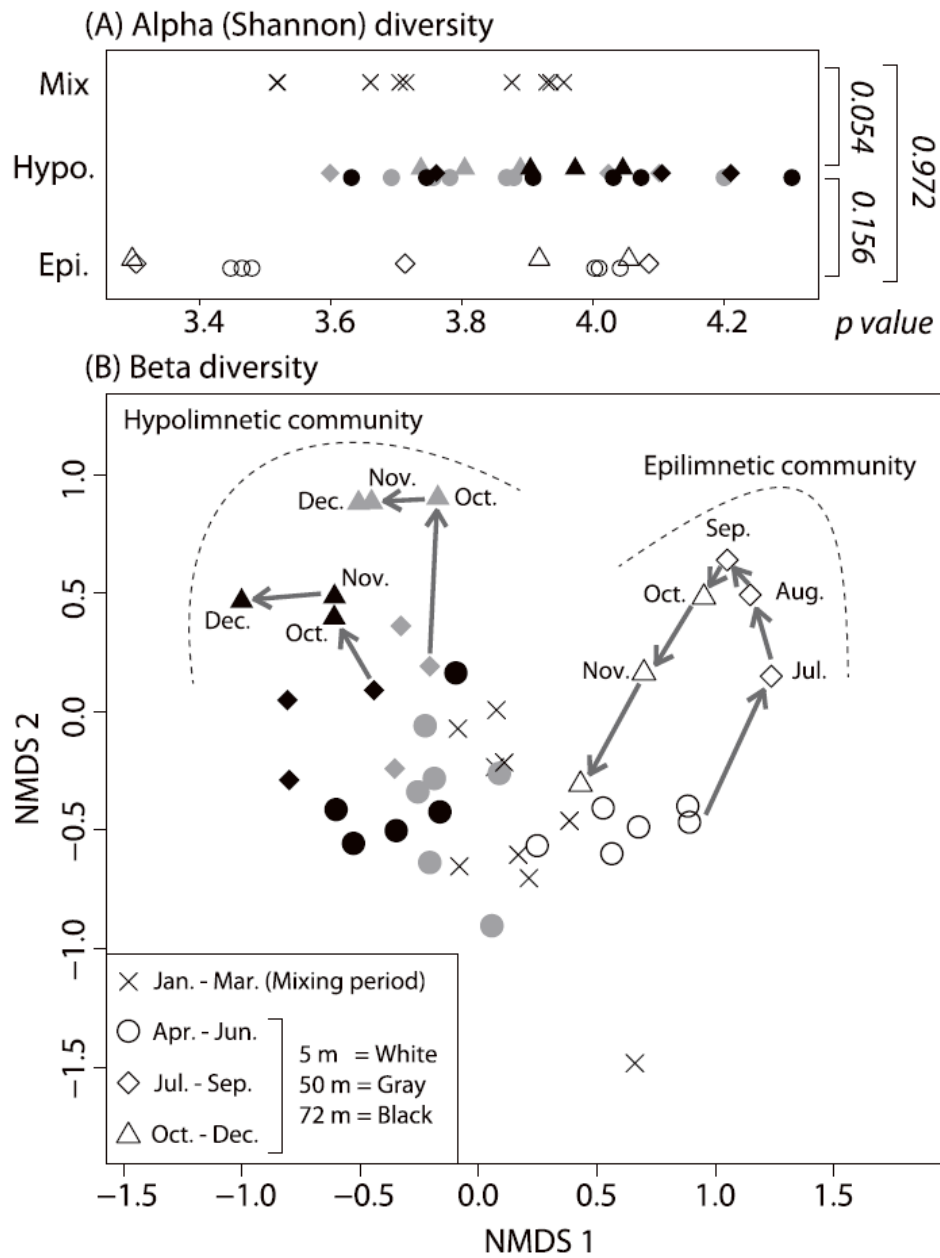


Figure 3-2

(A) Alpha (Shannon) diversity of 45 individual samples. Samples from the mixing period (Mix), the hypolimnion (Hypo.), and the epilimnion (Epi.) during stratification, were separately plotted. The differences between them were tested by the Wilcoxon rank sum test. The p values are shown on the right of the panel. (B) Beta diversity of 45 individual samples analyzed by non-metric multidimensional scaling (NMDS). Arrows indicate transitions of sequentially taken samples at each depth that characterizes the epilimnetic and hypolimnetic communities. Plot symbols and colors illustrate sampling depths and seasons.

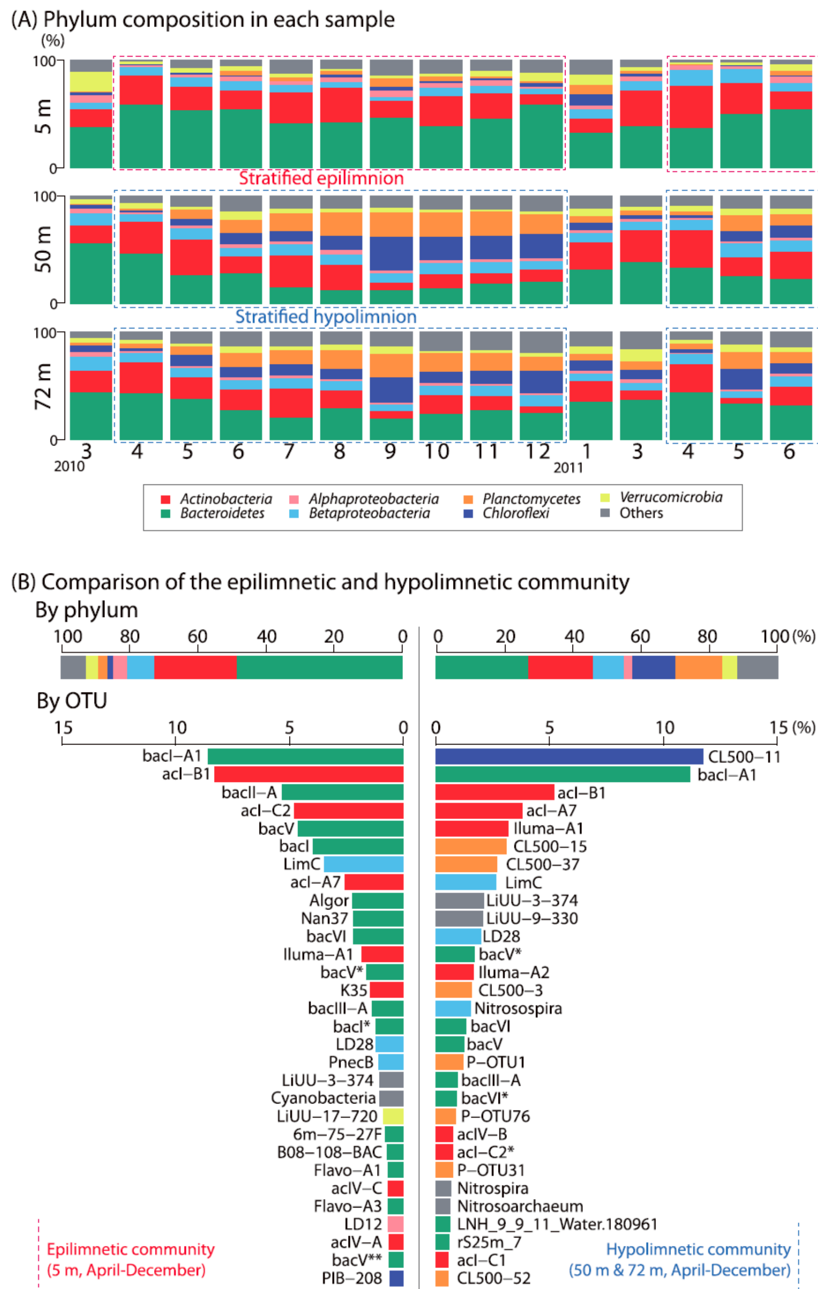


Figure 3-3

(A) Phylum-resolved community composition of individual samples, shown in the percentage to the total amplicon reads. Numbers on the horizontal axis indicate the sampling month. (B) The composition of the epilimnetic (left) and hypolimnetic (right) communities, composed of pooled data of each layer during stratification (highlighted by dashed rectangles in the panel A). A bar graph on the top shows phyla assignment in each community. Bars shown below indicate the proportion of the 30 predominant OTUs in each community. Bar colors indicate phyla to which individual groups were assigned. Asterisks in the group name distinguish the different OTUs assigned to the same group.

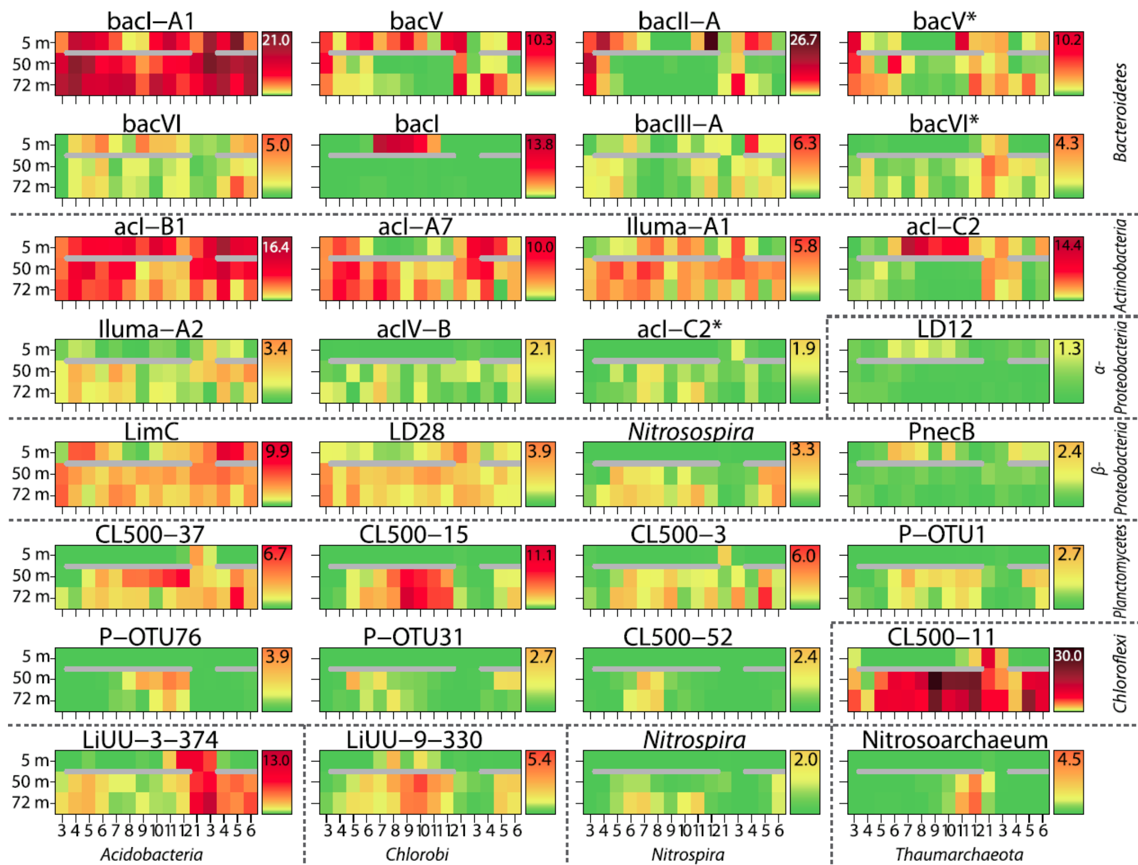


Figure 3-4

Spatiotemporal distribution patterns of the predominant phylogenetic groups, shown in the proportion (%) to the total amplicon read in each sample. In each panel, abundances of three depths × 15 months were indicated by colors shown in the three rows × 15 columns matrix. Colors in all heat maps are comparable (i.e., drawn to the same scale) while the color range in individual panels is shown on the right side of the matrix with the maximum value recorded in the group. Panels are arranged by phylum, separated by dashed lines. Asterisks in the group name distinguish the different OTUs assigned to the same group. Gray lines in the matrix illustrate the separation of the epilimnion and hypolimnion by a thermocline (April–December).

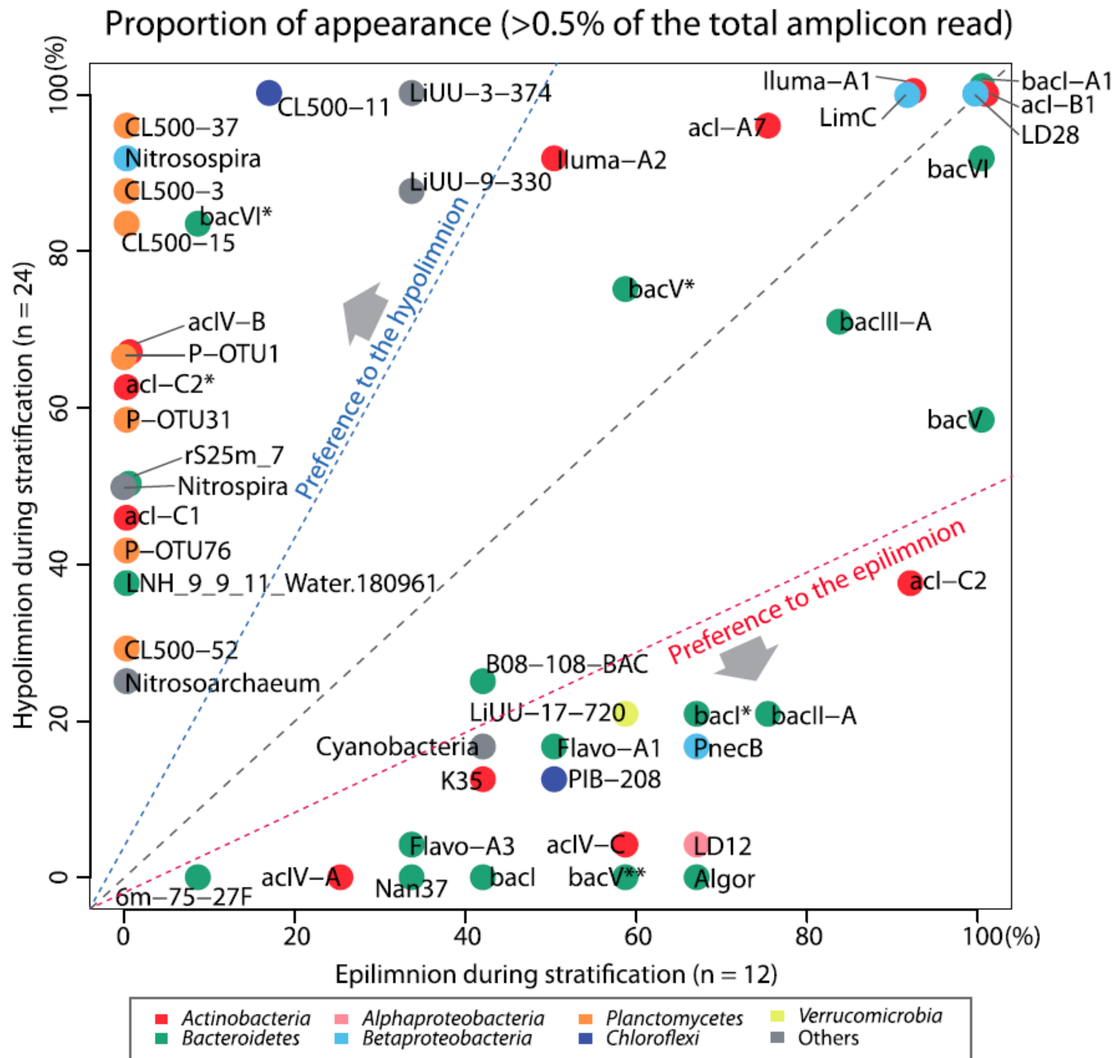


Figure 3-5

Proportion of appearance in each water layer of the 49 predominant groups. The horizontal and vertical axes indicate percentages from 12 epilimnion and 24 hypolimnion samples during stratification, respectively. Data points at the top left side suggest a preference for the hypolimnion and data points at the bottom right side suggest a preference for the epilimnion. Colors of the data points illustrate the phyla to which individual groups were assigned. Asterisks in the group name distinguish the different OTUs assigned to the same group.

Chapter 4: Ubiquity and quantitative significance of hypolimnion-specific bacterioplankton in deep freshwater lakes

Introduction

Bacterioplankton affiliated with the phyla *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* are globally predominant in freshwater systems (Zwart et al., 2002; Newton et al., 2011). However, these data are based on studies in surface waters, and members of other phyla could dominate the oxygenated hypolimnion. Among them, the *Chloroflexi* CL500-11 clade (Urbach et al., 2001, 2007; Okazaki et al., 2013; Denef et al., 2016) and the *Thaumarchaeota* Marine Group I (MGI) group (Urbach et al., 2001, 2007; Auguet et al., 2012; Vissers et al., 2013; Coci et al., 2015; Mukherjee et al., 2016) are the most investigated. The relatively large cell size (1–2 μm) and high abundance (>15% of all bacteria and archaea) of CL500-11 suggest their quantitative importance in the oxygenated hypolimnion (Chapter 2). MGI accounted for 8.7–19% of all bacterioplankton in the oxygenated hypolimnion of subalpine lakes (Callieri et al., 2016). They are ammonia-oxidizing archaea, and members in the oxygenated hypolimnion are affiliated with either *Nitrosopumilus* or *Candidatus* Nitrosoarchaeum (Coci et al., 2015). Other nitrifiers such as *Nitrosospira* and *Nitrospira* have also been found in the water column of the oxygenated hypolimnion (Mukherjee et al., 2016; Fujimoto et al., 2016). Moreover, high-throughput sequencing of the 16S rRNA gene partial amplicon has highlighted members of *Planctomycetes* (e.g., CL500-3, CL500-15, and CL500-37) as abundant lineages in the oxygenated hypolimnion (Rozmarynowycz, 2014). In the previous chapter, the existence of these hypolimnion-specific lineages was confirmed in Lake Biwa (Chapter 3). These inhabitants are not negligible components of the microbial food web and biogeochemical cycling in the deep pelagic freshwater ecosystem. However, it remains unknown how ubiquitously and abundantly they are distributed in the

oxygenated hypolimnion. Due to a lack of quantitative data, their ecological importance remains poorly understood.

The present study investigated the bacterioplankton community in the oxygenated hypolimnia of 10 deep freshwater lakes with a variety of geochemical characteristics. Community composition was investigated by 16S rRNA gene amplicon sequencing, and several representative members were microscopically characterized and quantified by CARD-FISH. Together with data previously collected from other deep lakes, the results allowed us to create the first comprehensive overview of the bacterioplankton community inhabiting the oxygenated hypolimnion of deep freshwater lakes and identify abundant and ubiquitous lineages. Moreover, analyses of their habitat preference and micro-diversification (oligotyping) facilitated hypotheses about their ecophysiological characteristics and potential diversified subpopulations.

Materials and Methods

Field sampling

Water samples were collected at pelagic sites in 10 deep freshwater lakes in Japan from August to December in 2015, including Lake Mashu, Kusharo, Toya, Inawashiro, Chuzenji, Sai, Motosu, Biwa, Ikeda, and T-Reservoir (hereafter, MA, KU, TO, IN, CH, SA, MO, BI, IK, and TR, respectively) (Fig. 4-1). Given that the total water volume in Japanese lakes is estimated to ca. 90 km^3 (Mori and Sato, 2015), the studied 10 lakes (total water volume = 50.0 km^3) comprise more than a half of the lake volume in the country. The profiles of the sampling locations are summarized in Table 4-1. In all, 3 to 13 depths were sampled in each lake, and the temperature and dissolved oxygen vertical profiles were measured using a CTD probe *in situ*. These lakes have permanently oxygenated hypolimnia and were thermally stratified when the sampling was carried out (Fig. 4-1). Total prokaryotic abundance was determined by microscopic enumeration of DAPI-stained cells (Porter and Feig, 1980) with at least 900 cells counted in each filter.

Partial 16S rRNA gene amplicon sequencing

In total, 33 DNA samples were collected (Fig. 4-1). Immediately after sampling, 70–300

mL the water collected was filtered through a 0.2 μm polycarbonate filter (47 mm diameter; Whatman, Maidstone, UK). The filter was stored at −20°C until DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). The V4 and V5 regions of the 16S rRNA gene were amplified using the modified 530F and 907R primers (Nunoura et al., 2009) and then an eight-base-pair DNA tag (for post-sequencing sample identification) and 454 adaptors were conjugated by second PCR, as described previously (Okazaki and Nakano, 2016). The PCR products from the samples were pooled in equimolar quantities and sequenced in the 1/8 regions of a sequencing reaction on the Roche 454 GS-FLX Titanium sequencer (Roche Science, Mannheim, Germany). The nucleotide sequence data are available in the Sequence Read Archive database under accession numbers DRX062810–DRX062842 (BioProject: PRJDB5151).

Analysis of sequencing reads

The sequence data were processed using the UPARSE pipeline (Edgar, 2013) following the author's instructions (http://www.drive5.com/usearch/manual/upp_454.html). I used a fastq_maxee value of 1.0, a truncated length of 350 bp, and an operational taxonomic unit (OTU) creation identity threshold of 97%. Thereafter, respective OTUs were taxonomically assigned with the SINA 1.2.11 (Pruesse et al., 2012) online tool (https://www.arb-silva.de/aligner/) referring to the 123 Ref database (Quast et al., 2013) and using the default parameter settings. Subsequently, non-prokaryotic OTUs (i.e., chloroplast, eukaryote, and unclassified domains) were removed. The resulting 96,149 reads were used for subsequent analyses.

Before calculating community diversity, coverage-based rarefaction (Chao and Jost, 2012) was applied. Reads were randomly discarded from each sample until coverage was <96.59% (i.e., slope of the rarefaction curve was >0.0341), which was the minimum value recorded among samples. Subsequently, alpha (the inversed Simpson index) and beta diversities (non-metric multidimensional scaling [NMDS] based on Bray–Curtis dissimilarity) were calculated. These analyses were performed using the vegan 2.4–0 package (Oksanen et al., 2016) in R 3.3.1 software (http://www.R-project.org/).

The OTUs were classified following the nomenclature proposed by Newton et al. (2011). Using the "--search" option in the SINA 1.2.11 (Pruesse et al., 2012) stand-alone tool, the representative sequences of the respective OTUs (generated by the UPARSE pipeline) were searched and classified against the original ARB (Ludwig et al., 2004) database provided by Newton et al. (2011). Dominant OTUs that failed to be classified by this procedure were named by the following schemes: If closely related (>99% identity) sequences with the prefixes "CL" (Urbach et al., 2001) or "LiUU-" (Eiler and Bertilsson, 2004) were in the public sequence database, their names were preferentially used. The *Planctomycetes* phylogenetic clades were newly defined for the collective descriptions (Fig. 4-S1). In other cases, the OTU was shown by its taxonomic affiliation (e.g., genus) based on the SILVA nomenclature.

The sequences were aligned using the SINA 1.2.11 (Pruesse et al., 2012) stand-alone tool against the SILVA 123 Ref NR 99 database to construct a phylogenetic tree (Quast et al., 2013). Maximum Likelihood trees were calculated by the RAxML 8.2.4 software (Stamatakis, 2014) with the GTR substitution model and the GAMMA rate heterogeneity model. The trees were drawn in MEGA 7 software (Kumar et al., 2016).

Oligotyping

Intra-OTU micro-diversity of representative lineages was analyzed by oligotyping, which facilitated detection of single nucleotide variation by excluding the effects of sequencing errors based on the Shannon entropy values (Eren et al., 2013). Following the author's instructions (http://merenlab.org/software/oligotyping/), the quality filtered FASTA file generated by the UPARSE pipeline was split at individual OTUs and aligned against the Greengenes (DeSantis et al., 2006) alignment database using the SINA 1.2.11 (Pruesse et al., 2012) stand-alone tool. Uninformative columns were removed by subsequently applying the "o-trim-uninformative-columns-from-alignment" and "o-smart-trim" scripts. Several rounds of oligotyping were repeated by manually choosing the most informative (i.e., the highest entropy) column until all oligotypes with > 100 reads exceeded the purity score of 0.90. The minimum substantive abundance parameter (option-M) was always set to 10.

CARD-FISH

CARD-FISH was performed based on Pernthaler et al. (2002) with some modifications as described previously (Okazaki et al., 2013). Specifically, the hybridization, amplification, and washing steps were carried out at 46°C. AlexaFluor 488 (Life Technologies, Carlsbad, CA, USA) was used as the tyramide-conjugated fluorescent dye. The oligonucleotide probes were designed previously for CL500-11 (Okazaki et al., 2013) and MGI (Coci et al., 2015), and newly designed for the other targets (Table 4-2). The probes were constructed using the “Design Probes” function in ARB 6.0.3 software (Ludwig et al., 2004) against the SILVA 123 Ref NR 99 database (Quast et al., 2013). Specificity of the probes was confirmed by an NCBI BLAST online search and the Test Probe 3.0 tool against the SILVA 123 Parc database (https://www.arb-silva.de/search/testprobe/). The probes designed in this study targeted the V4 or V5 regions of the 16S rRNA gene, which were targeted by amplicon sequencing. Since there could be probe-matching (false-positive) sequences that are not present in the database, I confirmed that the probes perfectly and exclusively match their target lineages in all of the sequenced reads. To enhance the fluorescent signal, oligonucleotide helpers (Fuchs et al., 2000) were used for several probes (Table 4-2). The helpers were designed to target the adjacent or opposite loci of the probe target site to loosen the secondary rRNA structure (Fuchs et al., 2000) by confirming that all probe-targets in the database were not mismatched with their corresponding helpers. The hybridization buffer contained 0.5 µg mL^{-1} probe and 0.1 µg mL^{-1} of each helper. The formamide concentration in the buffer was determined for each probe (Table 4-2) by testing a series of concentrations to obtain the best stringency (i.e., highest concentration without signal loss). The stringency test was performed in samples with a high read proportion of the target determined by amplicon sequencing. The hybridized filters were counterstained with DAPI and enumerated under an epifluorescence microscope. At least 300 DAPI-positive cells and the corresponding CARD-FISH-positive cells were enumerated three times per sample (the same filter piece). A negative NON338 probe control (Wallner et al., 1993) confirmed that no false-positive cells were present.

Results

16S rRNA gene partial amplicon sequencing generated 666 OTUs from the 96,149 reads originating from the 33 samples collected from the water columns of the 10 lakes. At the phylum-level phylogenetic resolution, *Actinobacteria* dominated throughout the water column, *Bacteriodetes* and *Betaproteobacteria* showed preference to the epilimnion, and *Chloroflexi* and *Planctomycetes* preferentially occurred in the hypolimnion (Figs. 4-2 and 4-S2). The epilimnetic and hypolimnetic communities were compared at the OTU level by averaging the samples from each layer (Fig. 4-2). The dominant members in the epilimnion were generally shared between the lakes (e.g., acI-B1, acI-A7, Lhab-A1, and bacI-A1). In addition to the lineages common to the epilimnion, *Chloroflexi* and *Planctomycetes* were also ranked as dominant OTUs in the hypolimnion (Fig. 4-2). CL500-11 alone accounted for most of the *Chloroflexi* reads, whereas *Planctomycetes* consisted of diverse OTUs (e.g., CL500-3, CL500-15, CL500-37, and plaI-A) (Fig. 4-2), which were affiliated with three classes in the phylum (Fig. 4-S1). The alpha diversity was higher in deeper samples in the lakes except for KU, CH and BI (Fig. 4-2). The beta diversity analysis (NMDS) clearly separated the hypolimnetic communities from the epilimnetic ones (Fig. 4-S3).

Vertical preference of the bacterioplankton in each OTU was examined based on the number of lakes where it accounted for >1% of all amplicon reads in each water layer (Fig. 4-3). This facilitated to separate epilimnion inhabitants, hypolimnion inhabitants and whole-layer inhabitants on the basis of the occurrence patterns (Fig. 4-3). Results produced by other abundance thresholds (>0.5% and >2%) are shown in Fig. 4-S4. Twenty-nine OTUs that were ubiquitous or specific to the hypolimnion were selected for further analysis (indicated by a gray dotted box in Fig. 4-3). While they showed preferences between the lakes, only few environmental parameters significantly explained the patterns (Fig. 4-4). The oligotyping analysis successfully detected conserved single-nucleotide intra-OTU variants (i.e., base positions with high entropy, which are not likely sequencing errors) (Fig. 4-4), and revealed that there were oligotypes that disproportionally distributed among the water layers or lakes (Fig. 4-5 and

Supplementary Data 4-1).

The newly constructed CARD-FISH probes targeted a monophyletic clade of the target lineages (Fig. 4-S5). Enumeration of the positive cells revealed that CL500-11 accounted for >10% of all prokaryotic cells in four lakes (maximum was 25.9% at 60 m in BI). CL500-3, CL500-37, and MGI respectively accounted for >3% in several lakes. CL500-15 were less abundant but still detectable, with a maximum percentage of 1.6% (Fig. 4-6). The cells detected in each target shared identical morphology between the lakes: CL500-11 were curved rods 1–2 µm long; CL500-3, CL500-37, and CL500-15 were cocci approximately 1 µm diameter; and MGI were rods around 1 µm long (Fig. 4-6). The cells were mostly planktonic, except for CL500-15, which were often found to be particle-associated; At 50 m in SA, 51% of the CL500-15 cells were particle-associated (determined by enumerating at least 300 positive cells for three times) (Fig. 4-6). The correlation between relative abundance determined by amplicon sequencing and CARD-FISH was significant for all targets, but the CARD-FISH estimates tended to be lower than those of amplicon sequencing (Fig. 4-S6). The ratio of CARD-FISH to amplicon sequence estimates was 0.57 on average (range, 0.14–1.02) for the five targeted lineages (Fig. 4-S6). The proportions of the respective targets in each sample (i.e., raw data for Fig. 4-6) are available in Supplementary Data 4-2.

Discussion

Structure of the bacterioplankton community in the oxygenated hypolimnion

The present study investigated lakes with a variety of environmental characteristics, ranging from a oligotrophic lake with the cold (4°C) hypolimnion (MA) to a mesotrophic lake with the relatively warm (11°C) hypolimnion (IK) (Fig. 4-1 and Table 4-1). Nevertheless, the beta-diversity analysis indicated that the bacterioplankton communities in the oxygenated hypolimnia were separated from the communities in the epilimnia (Fig. 4-S3), indicating that the oxygenated hypolimnion is an independent habitat for microbial communities. The hypolimnetic community consisted of members present across the water layers (whole-layer inhabitants), and members that

preferentially occurred in the oxygenated hypolimnion (hypolimnion inhabitants) (Fig. 4-3). The whole-layer inhabitants were composed of several commonly known freshwater bacterioplankton lineages (e.g., acI, Iluma-A1, Iluma-A2, Lhab-A1, LD28, and bacI-A1), whereas the hypolimnion inhabitants were represented by phyla that were not common to the epilimnion, including *Chloroflexi* CL500-11, members of *Planctomycetes* (e.g., CL500-3, CL500-15, CL500-37, and plaI-A), and *Ca.* Nitrosoarchaeum in *Thaumarchaeota* (Figs. 2 and 3). Their abundance and ubiquity in the hypolimnion of the studied lakes were demonstrated by the CARD-FISH analysis, showing that they collectively accounted for 1.5% (TR) to 32.9% (BI) of all bacterioplankton in the hypolimnion (Fig. 4-6 and Supplementary Data 4-2). The analysis also revealed that these groups were not always present in the studied lakes and were absent in some lakes (e.g., IN and KU for CL500-11, CH for CL500-3, and MO for CL500-37) (Figs. 4-3, 4-4, and 4-6), suggesting that they have respective habitat preferences.

Ubiquity, quantitative importance, and potential ecophysiology of the hypolimnion inhabitants

The predominance (~25% of all bacterioplankton) of planktonic CL500-11 cells in several lakes (Fig. 4-6) suggests that their resources are diffuse, abundant and ubiquitous. The metagenome-assembled genome and *in situ* transcriptional evidence of CL500-11 in Lake Michigan suggests their importance in peptide turnover (Denef et al., 2016). Peptides in aquatic systems are mainly derived from peptidoglycans in the bacterial cell wall (McCarthy et al., 2013; Nagata et al., 2003) and from proteins released by other bacteria (Tanoue et al., 1995) or phytoplankton (Nguyen and Harvey, 1997; Yamada et al., 2012). Previous studies in BI have collectively demonstrated that N-rich (by stoichiometry) or protein-like (by fluorescence properties) semi-labile dissolved organic matter (DOM) derived from autochthonous phytoplankton production that accumulates in the hypolimnion is slowly remineralized during stratification (Kim et al., 2006; Maki et al., 2010; Thottathil et al., 2013). Thus, it is possible that CL500-11 is scavenging protein-like debris that accumulates in the lake due to its relatively recalcitrant nature. Given that the water retention time of a system affects DOM composition (Kellerman et

al., 2014; Catalán et al., 2016) and that autochthonous dissolved proteins can accumulate in a lake even at centennial time scales (Goldberg et al., 2015), I expected that lakes with a longer water retention time would contain more bacterioplankton lineages specialized to consume relatively recalcitrant DOM. In the present study, the water retention time of the lakes ranged from 0.27 years (TR) to 200 years (MA) (Table 4-1). However, most of the hypolimnion inhabitants, including CL500-11, were not distributed in a manner that was associated with water retention time (Fig. 4-4). As CL500-11 is a large cell (Fig. 4-6), protistan size-selective grazing (Pernthaler, 2005) may be a factor controlling CL500-11 dynamics, and little information is available on the grazer communities inhabiting the deep oxygenated hypolimnion (Masquelier et al., 2010; Mukherjee et al., 2015). These assumptions should be verified by future studies, given their ubiquity and quantitative importance in deep freshwater systems. Indeed, the dominance of CL500-11 has been reported in the two largest deep freshwater systems on Earth, the Laurentian Great Lakes (Rozmarynowycz, 2014; Denef et al., 2016) and Lake Baikal (Kurilkina et al., 2016).

CL500-3 and CL500-37 were the two most abundant *Planctomycetes* in the present study (Fig. 4-6). Each was affiliated with their respective phylogenetic clade in the class *Phycisphaerae* (Fig. 4-S1). Aquatic *Planctomycetes* are often associated with algal blooms (Morris et al., 2006; Pizzetti et al., 2011), and genomic evidence indicates their potential to aerobically consume sulfated polysaccharides derived from algae (Glöckner et al., 2003; Woebken et al., 2007; Erbilgin et al., 2014). In a marine study, sequences closely related to CL500-3 were enriched in DNA extracted from bacterioplankton that assimilate protein secreted by phytoplankton (Orsi et al., 2016). Consequently, it can be hypothesized that CL500-3 and CL500-37 consume polysaccharides or proteins derived from phytoplankton. The two lineages did not always co-occur, and disproportional dominance of CL500-3 was found in MO and TR, and of CL500-37 in CH and IN (Fig. 4-6). Amplicon sequencing data taken from the oxygenated hypolimnion in Lake Michigan (Fujimoto et al., 2016) showed that only CL500-37 was abundant (Fig. 4-S7). These observations indicate that the ecological niches of CL500-3

and CL500-37 are not the same. Given that algal exudates from different phytoplankton species select different bacterial communities (Šimek et al., 2011; Paver et al., 2013), the difference might be attributable to differences in the phytoplankton species in a lake. It should also be noted that their closely related sequences were not necessarily retrieved from the oxygenated hypolimnion and have been found in an Antarctic lake (Karlov et al., 2016), arctic lake (Ntougias et al., 2016), and Baltic Sea ice (Eronen-Rasimus et al., 2015) (Fig. 4-S5A). More information is needed to elucidate the ecophysiological characteristics of these widespread and abundant yet largely overlooked bacterial lineages.

The CL500-15 clade belonged to the uncultured OM190 class (Fig. 4-S1) with only three sequences in the database: two from deep freshwater lakes (Urbach et al., 2001; Pollet et al., 2011) and one from deep sea sediment (Zhang et al., 2013) (Fig. 4-S5B). Another sequence was reported from the littoral water of Lake Baikal (Parfenova et al., 2013). In the present study, CL500-15 was detected in half of the lakes (Fig. 4-3), indicating that they are one of the most common lineages in the oxygenated hypolimnion. The CARD-FISH analysis revealed a high proportion of particle-associated cells and microscopic observations revealed that the particles were not cells but rather looked like transparent exopolymer particles (TEP), which are gel-like sticky particles mainly composed of polysaccharides (Passow, 2002). Members of the OM190 class in marine systems have been preferentially detected in the particle-associated fraction (Salazar et al., 2015; Bižić-Ionescu et al., 2015), suggesting that their particle-associated form is preserved across the class. As particle-associated bacteria can contribute disproportionally to total bacterial activity (Lemarchand et al., 2006; Grossart et al., 2007), CL500-15 may play a substantial role in substrate remineralization in the oxygenated hypolimnion.

Other representative *Planctomycetes* were affiliated with class *Planctomycetacia* (plaI-A–F) (Fig. 4-S1). They usually had a smaller proportion of reads than those of CL500-3, CL500-37, and CL500-15 (Fig. 4-2). However, plaI-A was the most represented *Planctomycetes* based on the read proportion in the oxygenated hypolimnion

of MA (Fig. 4-2) and Lake Michigan (Fig. 4-S7). Each of the other members of plaI (e.g., plaI-B [CL500-52], plaI-D, and plaI-E) showed their respective distribution patterns between the lakes (Fig. 4-4). Overall, *Planctomycetacia* (plaI group) was generally less ubiquitous and abundant, but more diverse than the other two classes (i.e., *Phycisphaerae* and OM190) inhabiting the oxygenated hypolimnion.

In the present study, the MGI group was detected only from two lakes (BI and IK) (Fig. 4-4), and their maximum percentage determined by CARD-FISH was 3.8% (Fig. 4-6). These numbers are lower than those of a previous study that detected 8.7–19% MGI in the oxygenated hypolimnion of all six subalpine lakes investigated (Callieri et al., 2016). In the present study, MGI was exclusively affiliated with *Ca.* Nitrosoarchaeum (Blainey et al., 2011), but *Nitrosopumilus*, another predominant MGI member in the oxygenated hypolimnion (Berdjeb et al., 2013; Vissers et al., 2013; Coci et al., 2015), was not detected. *Ca.* Nitrosoarchaeum has been reported in the oxygenated hypolimnion of Crater Lake (Urbach et al., 2001, 2007), Lake Redon (Auguet et al., 2012), and Lake Superior (Mukherjee et al., 2016), which are oligotrophic lakes with a hypolimnetic temperature of 4°C. The occurrence of *Ca.* Nitrosoarchaeum in BI and IK, which are mesotrophic lakes with the hypolimnetic temperatures of 8 and11°C, respectively (Fig. 4-1), revealed their broad habitat spectrum. In other lakes (e.g., CH and TR), ammonia-oxidizing bacteria, *Nitrosospira*, were detected in the oxygenated hypolimnion (Fig. 4-4), in agreement with the previously suggested niche separation between ammonia-oxidizing archaea and bacteria (Coci et al., 2015; Mukherjee et al., 2016). Another nitrifier, *Nitrospira*, was also detected in the oxygenated hypolimnion (Fig. 4-4), in line with previous studies (Small et al., 2013; Okazaki and Nakano, 2016). However, no nitrifiers were detected in the three northern lakes (MA, KU, and TY) (Fig. 4-4). Notably, their absence should not be concluded by the present data, as the nitrifier community can shift over seasons (Okazaki and Nakano, 2016). Nevertheless, the results indicate the potential diversity of the nitrification systems in the oxygenated hypolimnion, yet information remains scarce to conclude the cause and effects of the diversity.

The discussion above is based on the assumption that each of the

bacterioplankton lineages prefer their particular suitable habitat. However, it is also possible that the occurrence of the members is controlled by occurrence of other lineages, as bacterioplankton often have streamlined genomes and are dependent on each other for lost metabolic functions (Morris et al., 2012; Garcia et al., 2015; Mas et al., 2016). In the present study, many pairs of hypolimnion specialists showed correlating distribution patterns between the lakes (Fig. 4-S8). For example, CL500-3 was positively correlated with CL500-37 and *Ca*. Nitrosoarchaeum, and negatively correlated with *Nitrosospira* (Fig. S4-8). Although these results do not directly support an interaction, they suggest that some hypolimnion specialists are presumably co-occurring or sharing similar ecological niches.

Notable but less represented lineages

Although several lineages originally described for Crater Lake (with the prefix "CL") (Urbach et al., 2001) were identified as representative hypolimnion inhabitants (Figs. 4-3 and 4-4), several other members dominant in Crater Lake were not highly represented in the present study: CL120-10 of *Verrucomicrobia*, CL0-1 of *Armatimonadetes*, and CL500-9 of *Chloroflexi*. It is plausible that further investigations in lakes on other continents or those with depths > 250 m will detect bacterioplankton not found in the present study. Methanotrophs, such as *Methylococcaceae* and *Methylocystaceae*, also accounted for a very minor fraction of all amplicon reads. A more intriguing result is the limited distribution and low relative abundance of LD12 (Figs. 4-2 and 4-3), which is one of the most dominant and ubiquitous freshwater bacterioplankton (Zwart et al., 1998; Newton et al., 2011; Salcher et al., 2011a). Notably, data produced using another sequencing platform (Miseq) from a part of the DNA samples used in the present study showed a higher read proportion of LD12 (S. Fujinaga, personal communication), despite the fact that both analysis used PCR primers that perfectly matched the LD12 16S rRNA gene sequence. Although the reason behind this discrepancy is unknown, it is possible that the relative abundance of LD12 was underestimated in the present study, which, in turn, might have overestimated other lineages among the reads, as in the discrepancy with the CARD-FISH results (Fig. 4-S6). Direct cell enumeration using FISH should be

considered an accurate abundance estimate.

Intra-OTU diversification revealed by the oligotyping analysis

The oligotyping analysis revealed the intra-OTU diversification of the whole-layer inhabitants (Fig. 4-4). Some of the oligotypes were disproportionally distributed among depths or lakes (Fig. 4-5 and Supplementary Data 4-1), suggesting that their ubiquity was achieved collectively by heterogeneous oligotypes that specialized in a respective niche. Such cryptic micro-diversification within a ubiquitous freshwater lineage with an almost identical 16S rRNA gene sequence has previously been reported in *Limnohabitans* (Kasalický et al., 2013; Jezbera et al., 2013) and *Polynucleobacter* (Jezbera et al., 2011; Hahn et al., 2015, 2016). These studies reported diversification between habitats with different temperatures, pHs, organic and inorganic substrate availability, and geography. Recently, a horizontal oligotype profile in Lake Michigan indicated oligotype diversification within predominant bacterioplankton lineages between estuarine and pelagic sites (Newton and McLellan, 2015). The present study discovered micro-diversification between the epilimnion and hypolimnion, suggesting the presence of hypolimnion-specific subpopulations within the common freshwater bacterioplankton (e.g., acI-B1, acI-A7; Fig. 4-5). Since oligotypes may not always reflect ecologically or evolutionarily cohesive populations (Berry et al., 2017), further comparative studies of individual oligotypes are required to clarify their diversification to the respective water layers, which differ considerably in temperature, substrate availability, and grazing pressure.

The low oligotype diversity of several hypolimnion inhabitants (e.g., CL500-11, CL500-3, CL500-37; Fig. 4-4) was intriguing, as I expected that the oligotype would be diverse between the hypolimnia of different lakes, which are physically isolated and differ in physicochemical properties (Table 4-1). The occurrence of CL500-3 in TR (Fig. 4-6), which is a reservoir constructed just 10 years before the sampling, support the idea that hypolimnion inhabitants migrate between lakes; thus, diversification is limited. On the other hand, diverse sequence types are likely present at least on a continental scale, as CL500-11 from North American lakes have a conserved single-nucleotide difference

from the Japanese ones, whereas European ones have both sequence types (Fig. 4-S9).

Conclusion

This study provides the first comprehensive overview of the bacterioplankton community inhabiting the oxygenated hypolimnion by investigating 10 deep freshwater lakes with a variety of environmental characteristics. Partial 16S rRNA gene amplicon sequencing and CARD-FISH discovered the presence and quantitative significance of the hypolimnion specific lineages, represented by *Chloroflexi*, *Planctomycetes*, and *Thaumarchaeota*. An oligotyping analysis suggested the presence of hypolimnion-specific subpopulations within the whole-layer inhabitants (e.g., acI-B1, acI-A7). These results revealed the ubiquity and quantitative significance of bacterioplankton lineages inhabiting the oxygenated hypolimnion of deep freshwater lakes. Collectively, the present study provides valuable information for further studies on deep freshwater ecosystems and motivate future works to focus on ecophysiological characteristics of the individual bacterial lineages.

Tables and Figures

Table 4-1

Profiles of the sites studied. Data were collected from Mori and Sato (2015) and the Japanese Ministry of the Environment public water database.

	Max. Depth (m)	Depth of sampling point (m)	Surface area (km^2)	Water Volume (10^6 m^3)	Surface Altitude (m)	Lake Origin	Water Retention Time (year)	Trophic Status	pH*	Total Nitrogen (mg/L)*	Total Phosphorus (mg/L)*	Sampling Location	Sampling date
Mashu (MA)	211.4	211	19.2	2640	351	Caldera	200	Oligotrophic	7.0–8.0	0.06–0.08	<0.003–0.004	43°35'16.0"N , 144°31'50.0"E	2015.8.26–29
Kusharo (KU)	117.5	117	79.6	2261	121	Caldera	12	Oligotrophic	7.0–7.5	0.07–0.24	<0.003–0.004	43°35'26.0"N , 144°19'48.0"E	2015.8.25
Toya (TO)	179.7	174	70.7	8272	84	Caldera	9	Mesotrophic	7.1–7.8	0.24–0.38	0.003–0.01	42°36'15.1"N , 140°49'07.7"E	2015.9.24
Inawashiro (IN)	93.5	92	103.3	5320	514	Tectonic	5.4	Oligotrophic	6.6–7.0	0.17–0.34	<0.003–0.004	37°28'27.6"N , 140°05'40.5"E	2015.10.30
Chuzenji (CH)	163	163	11.8	1116	1269	Dammed	5.9	Oligotrophic	7.7–8.8	0.07–0.27	<0.003–0.009	36°43'55.7"N , 139°28'25.9"E	2015.10.21
Sai (SA)	71.7	71	2.1	81	900	Dammed	1.6	Oligotrophic	7.4–8.8	0.06–0.32	0.003–0.008	35°29'49.5"N , 138°41'09.5"E	2015.9.8
Motosu (MO)	121.6	120	4.7	319	900	Dammed	6.5	Oligotrophic	6.8–7.9	0.15–0.25	<0.003–0.006	35°28'06.5"N , 138°34'57.8"E	2015.9.7
T-Reservoir (TR)	121	121	13	660	390	Artificial Reservoir	0.27	Mesotrophic	7.2–9.2	0.18–0.46	<0.003–0.008	35°40'26.0"N , 136°29'57.0"E	2015.9.16
Biwa (BI)	103.8	97	670.3	27616	85	Tectonic	5.5	Mesotrophic	7.7–8.4	0.15–0.32	0.005–0.009	35°23'21.0"N , 136°07'51.0"E	2015.12.9
Ikeda (IK)	233	233	10.9	1368	66	Caldera	33.5	Mesotrophic	7.1–8.6	0.14–0.32	<0.003–0.006	31°14'14.0"N , 130°33'52.0"E	2015.9.17

*Annual range recorded in the epilimnion.

Table 4-2

Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) probes, helpers, and formamide concentrations (at 46°C) used in this study. Helpers are indicated by the suffix "h" in their names.

Probe (Helper)	Sequence	Length (base)	Formamide	Target	Reference
CLGNS_584	GCCGACTTGCCCAACCTC	18	20%	CL500-11	Okazaki et al. (2013)
CLGNS_567h	CTACACGCCCTTTACGCC	18			
CL500-3_829	CGTTCAACCCCCTCGACC	18	20%	CL500-3	This study
CL500-3_847h	ATCAGTTTTCCTACGGCCGAGAAGG	25			
CL500-3_804h	AAGTGCGCATCGTTTACGGCGTGGA	25			
CL500-37_658	CGTTCCACCCGCCTCTACCTG	21	30%	CL500-37	This study
CL500-15_826	ACAGGGAGACCCCAAACTAG	20	30%	CL500-15	This study
CL500-15_846h	TCACTTTTGTTTCGCCTGGGATCCC	25			
CL500-15_801h	TGCCCATCGTTTACAGCTAGGACTA	25			
MGI_535	TCCTGACCACTTGAGGTGCTGG	22	25%	MGI	Coci et al. (2015)

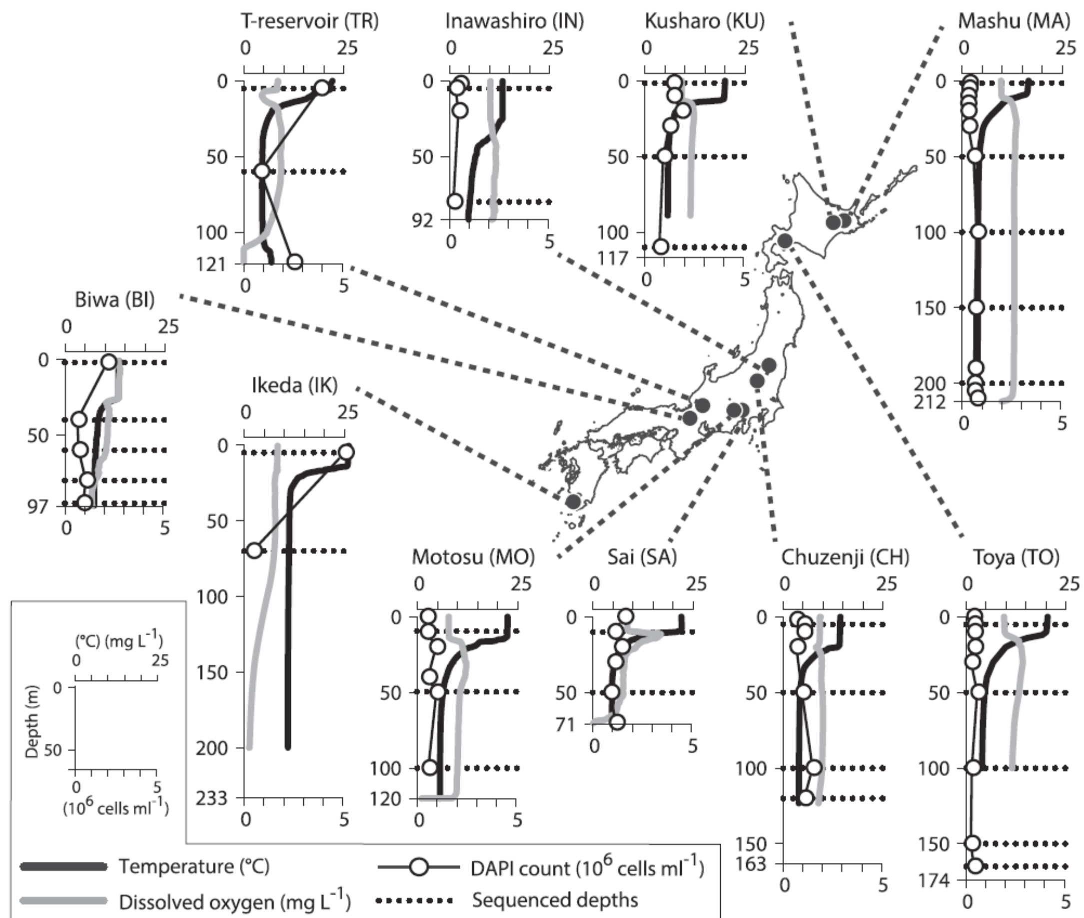


Figure 4-1

Locations and vertical profiles (water temperature, dissolved oxygen, and DAPI positive cells) of the sampling sites.

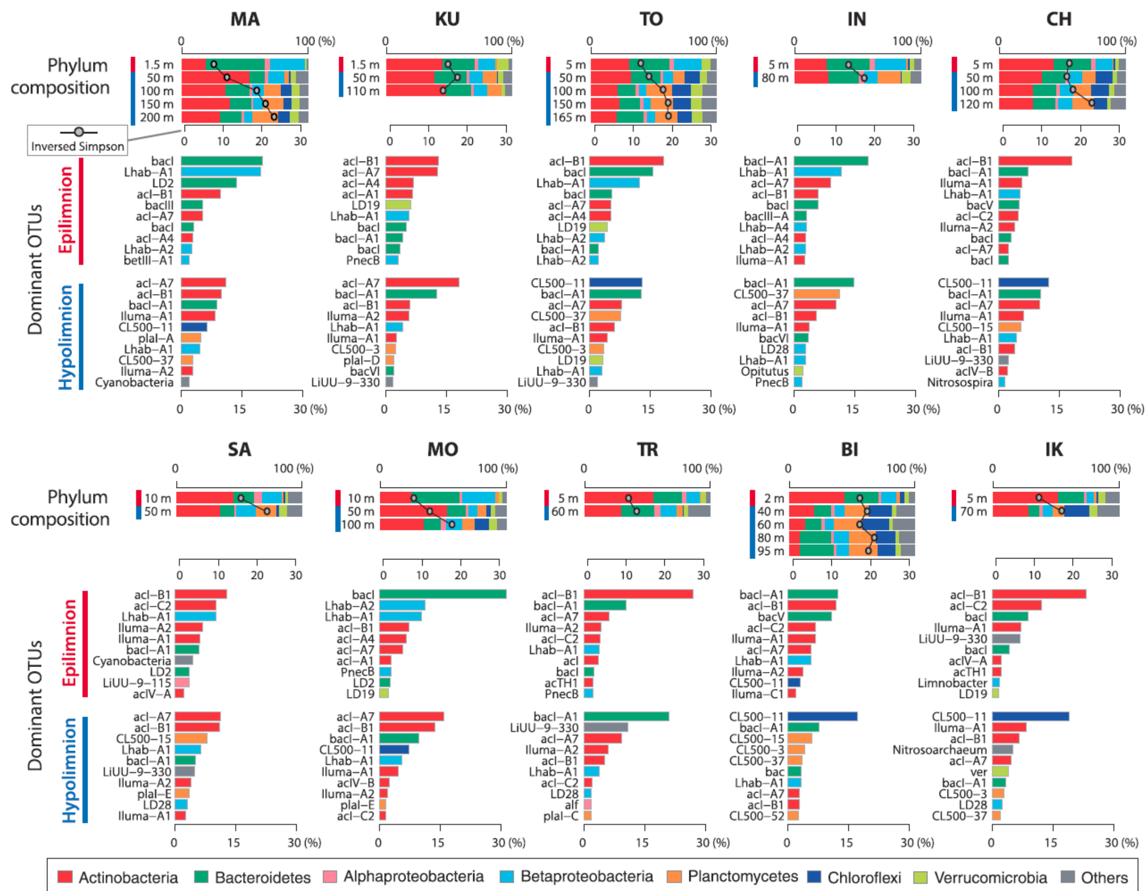


Figure 4-2

Composition of the 16S rRNA gene amplicon reads. The top panel (band graphs) displays phylum-resolved community composition at each depth for each lake, with an overwritten line graph indicating the alpha diversity (the inverse Simpson index). The two lower panels show the 10 dominant operational taxonomic units (OTUs) in the epilimnion and hypolimnion, composed of averaged data for each layer (the depths averaged are illustrated by red and blue lines in the top panel). Bar colors indicate phyla to which individual OTUs were assigned.

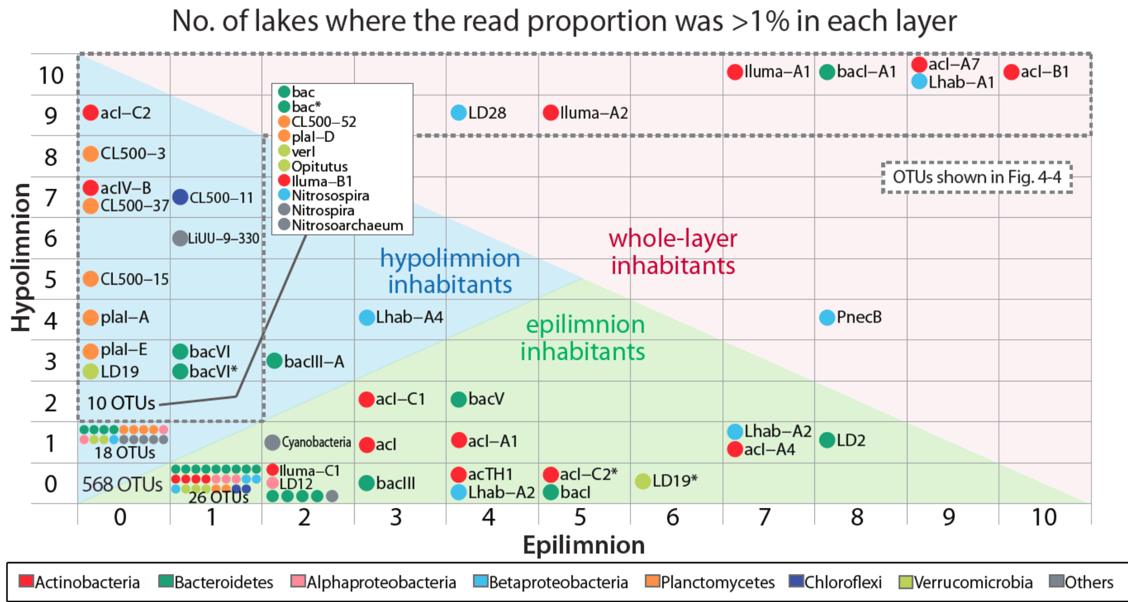


Figure 4-3

Vertical preferences of individual operational taxonomic units (OTUs), mapped by the number of lakes where individual OTUs accounted for >1% of all amplicon reads in each water layer. Data for the hypolimnion were generated by averaging the data at multiple depths in the hypolimnion. The OTUs were categorized into three groups (epilimnion-, hypolimnion-, and whole-layer inhabitants) based on their distribution patterns. A gray dotted box highlight the 29 OTUs selected for further analyses shown in Fig. 4-4. Point color indicates the phylum to which an individual OTU was assigned. Asterisks in the group name distinguish the different OTUs assigned to the same group. Results produced by other abundance thresholds (>0.5% and >2%) are shown in Fig. 4-S4.

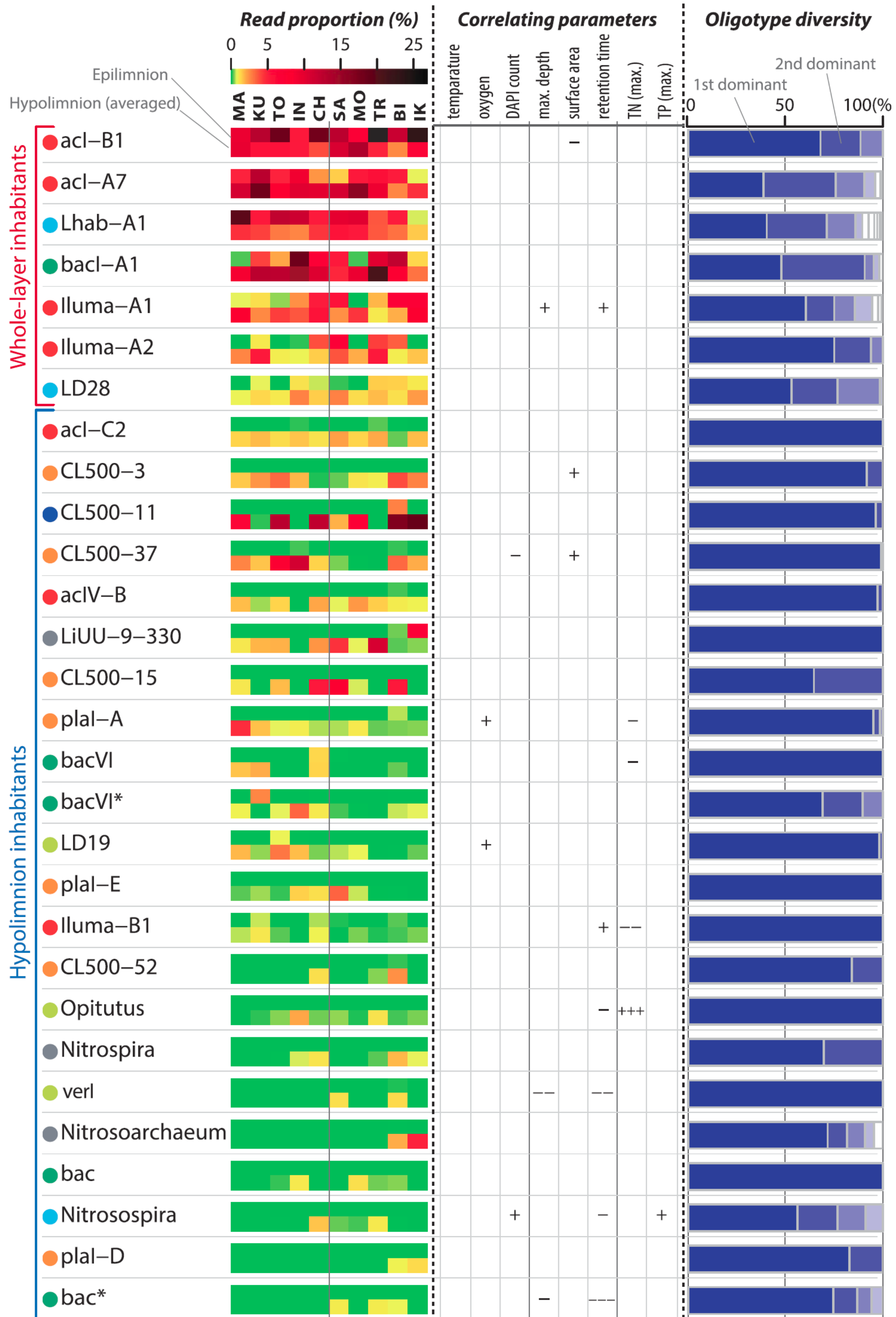

Read proportion (%)
0 5 15 25
Epilimnion
Hypolimnion (averaged)
MA KU TO IN CH SA MO TR BI IK
Correlating parameters
temparature
oxygen
DAPI count
max. depth
surface area
retention time
TN (max.)
TP (max.)
Oligotype diversity
2nd dominant
1st dominant
0 50 100(%
Whole-layer inhabitants
Hypolimnion inhabitants
acl–B1
acl–A7
Lhab–A1
bacl–A1
Iluma–A1
Iluma–A2
LD28
acl–C2
CL500–3
CL500–11
CL500–37
aclV–B
LiUU–9–330
CL500–15
plal–A
bacVI
bacVI*
LD19
plal–E
Iluma–B1
CL500–52
Opitutus
Nitrospira
verI
Nitrosoarchaeum
bac
Nitrosospira
plal–D
bac*

Figure 4-4

Distribution patterns (left column), correlating environmental parameters (center), and oligotype diversity (right) of the hypolimnion inhabitants. The distribution pattern is illustrated by the read proportion to total amplicon reads. The hypolimnion data were generated by averaging the data at multiple depths in the hypolimnion. The correlation between read proportion and environmental parameters in the hypolimnion was evaluated by Spearman's test. For a positive correlation, +++, $p < 0.005$; ++, $p < 0.01$; +, $p < 0.05$. For a negative correlation, "–" was shown instead of "+". For TN and TP, maximum values recorded in the lake were used (Table 4-1). The right column (band graphs) indicates composition of the oligotypes among all amplicon reads assigned to each OTU. Point colors indicate phyla to which individual OTUs were assigned (See Figs. 4-2 and 4-3 for legend). Asterisks in the group name distinguish the different OTUs assigned to the same group.

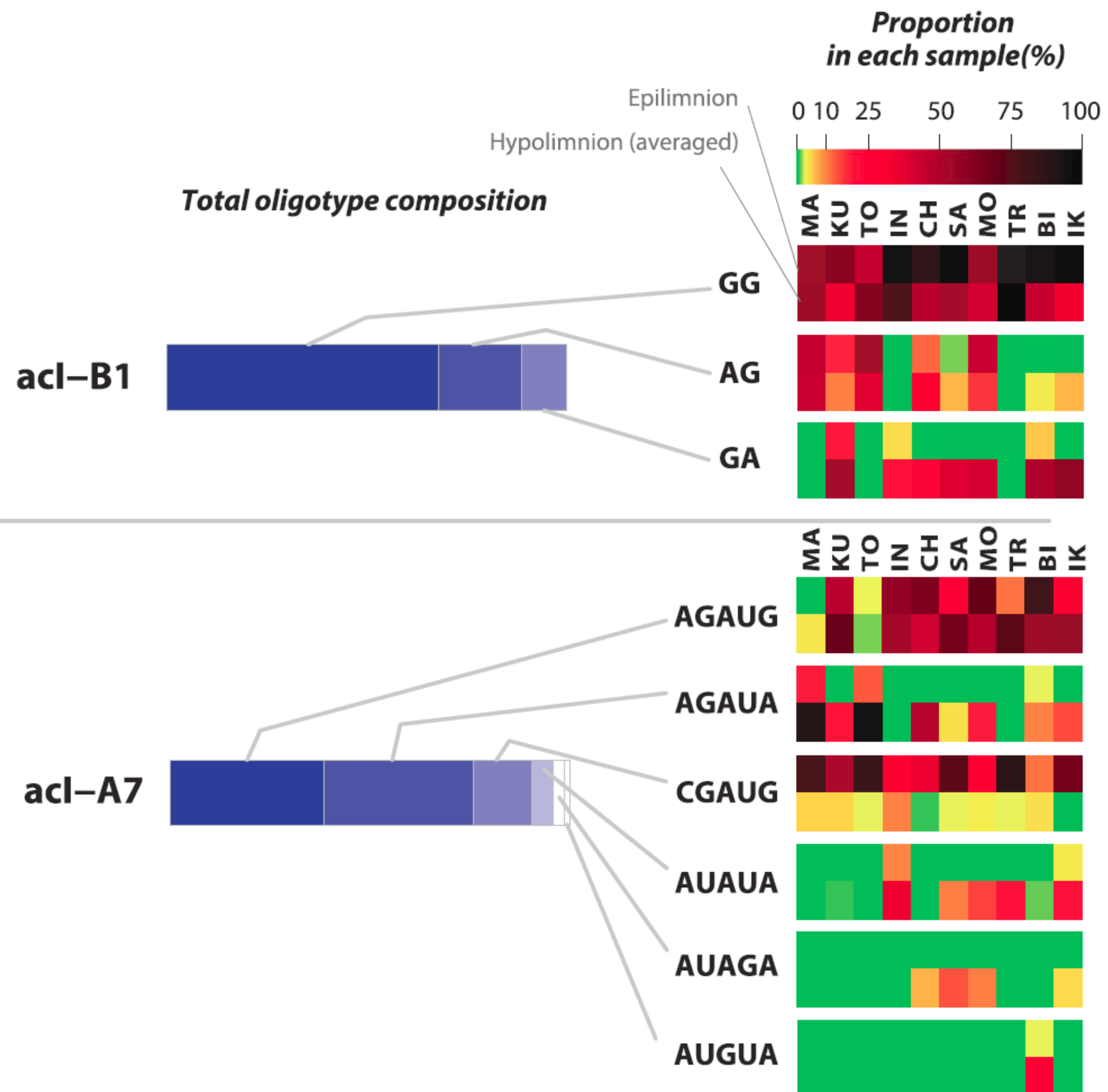


Figure 4-5

Composition and distribution of oligotypes for two abundant whole-layer inhabitants, acI-B1 and acI-A7. The left bars indicate the total oligotype composition in the present study, whereas right panels indicate the proportion of the respective oligotypes in each sample. Data for the hypolimnion were generated by averaging the data at multiple depths in the hypolimnion. The complete dataset including all OTUs is available in Supplementary Data 4-1.

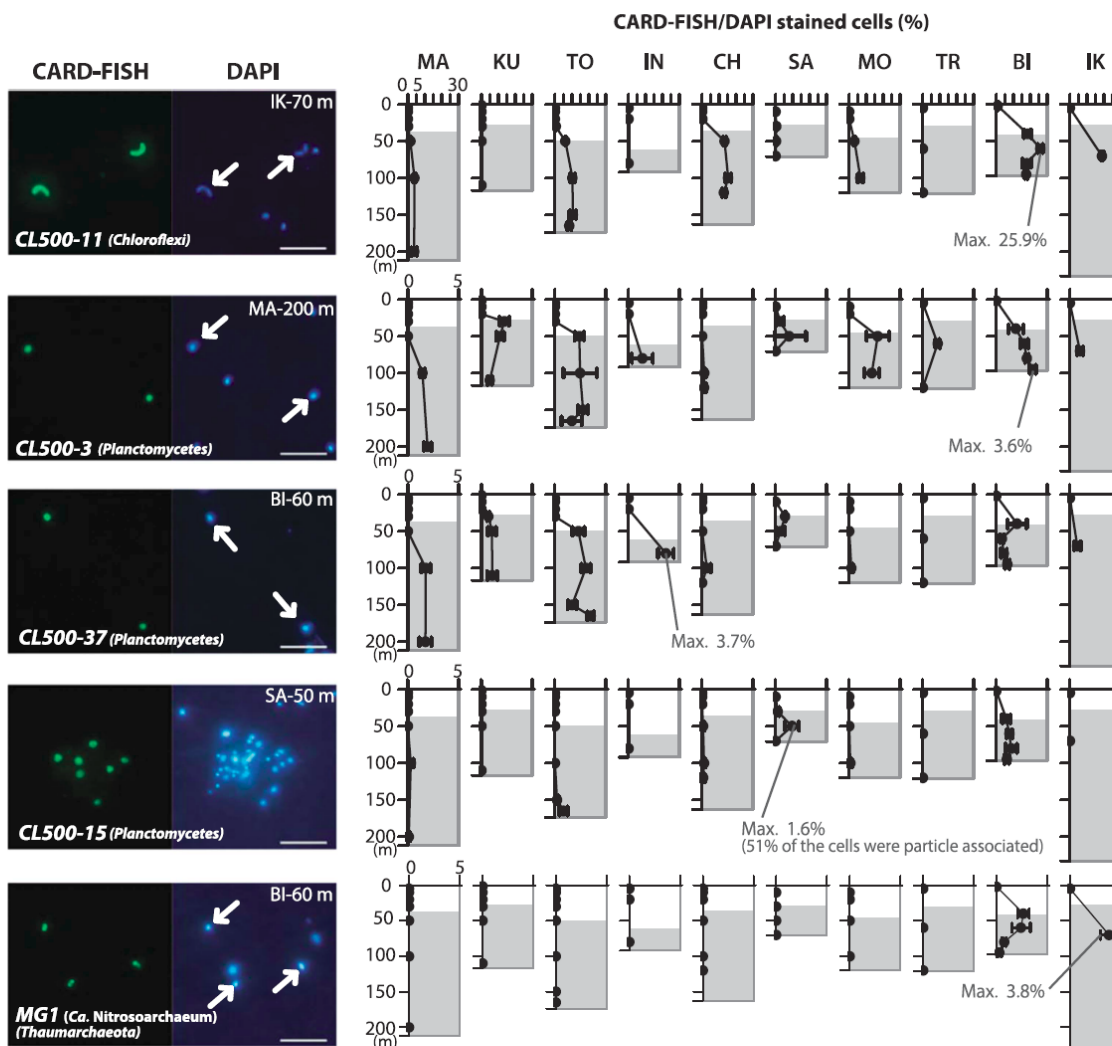


Figure 4-6

Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) images and the enumeration results. Positive cells and the corresponding DAPI-stained image are shown in each micrograph. Arrows in the DAPI-image indicate cells with CARD-FISH-positive signals. Scale bar = 5 μm. The line graphs show percentages of CARD-FISH-positive cells to DAPI-positive cells, and the error bars indicating the standard deviation determined by triplicate enumeration of an identical filter. The maximum value recorded for each lineage is designated. Gray background illustrates the hypolimnion (i.e., below the thermocline). The raw data are available in Supplementary Data 4-2.

Supplementary Information

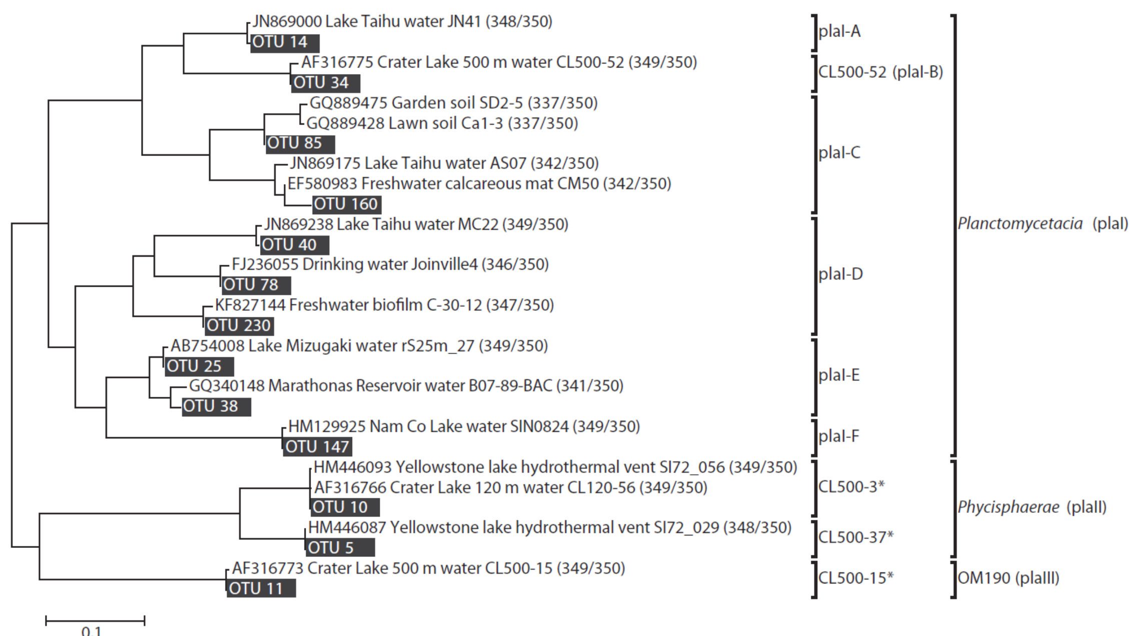


Figure 4-S1

Maximum likelihood phylogenetic tree of the major (>1% of all amplicon reads in at least one sample) *Planctomycetes* operational taxonomic units (OTUs). The closest relatives in the SILVA Ref NR database were used as reference. The topology of the tree is identical to that of the SILVA Ref NR guide tree. Numbers in parenthesis shows identity to the query (350 bp) OTU sequence.

*Detailed trees for CL500-3, CL500-15, and CL500-37 are available in Fig. 4-S5.

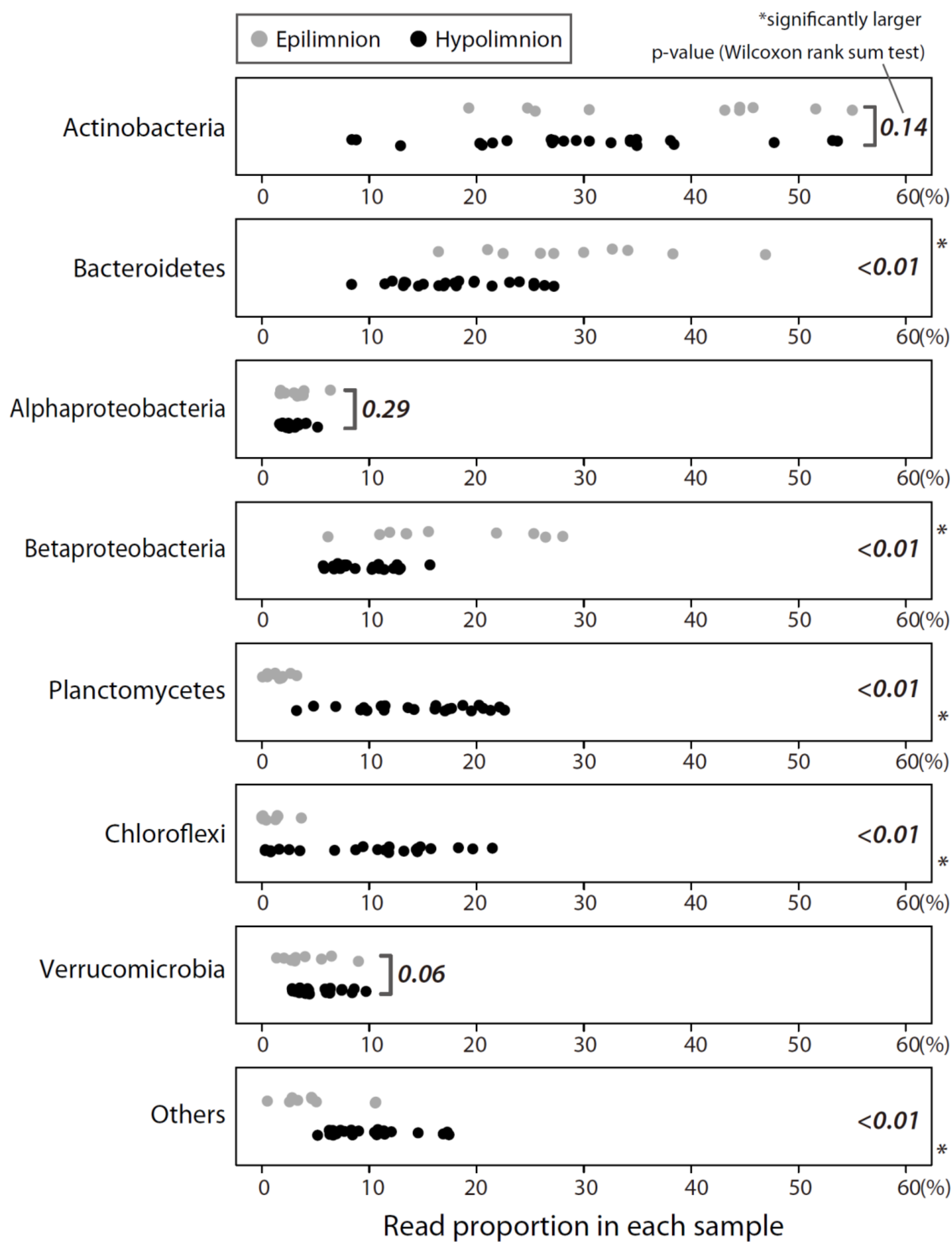


Fig. 4-S2

Statistical tests for the differences between epilimnetic and hypolimnetic read proportions for individual phylum (data shown in the top panels in Fig. 4-2), performed by the Wilcoxon rank sum test. Each point represents individual sample and an asterisk indicates water layer showing significantly ($p < 0.01$) higher read proportion than that of the other layer.

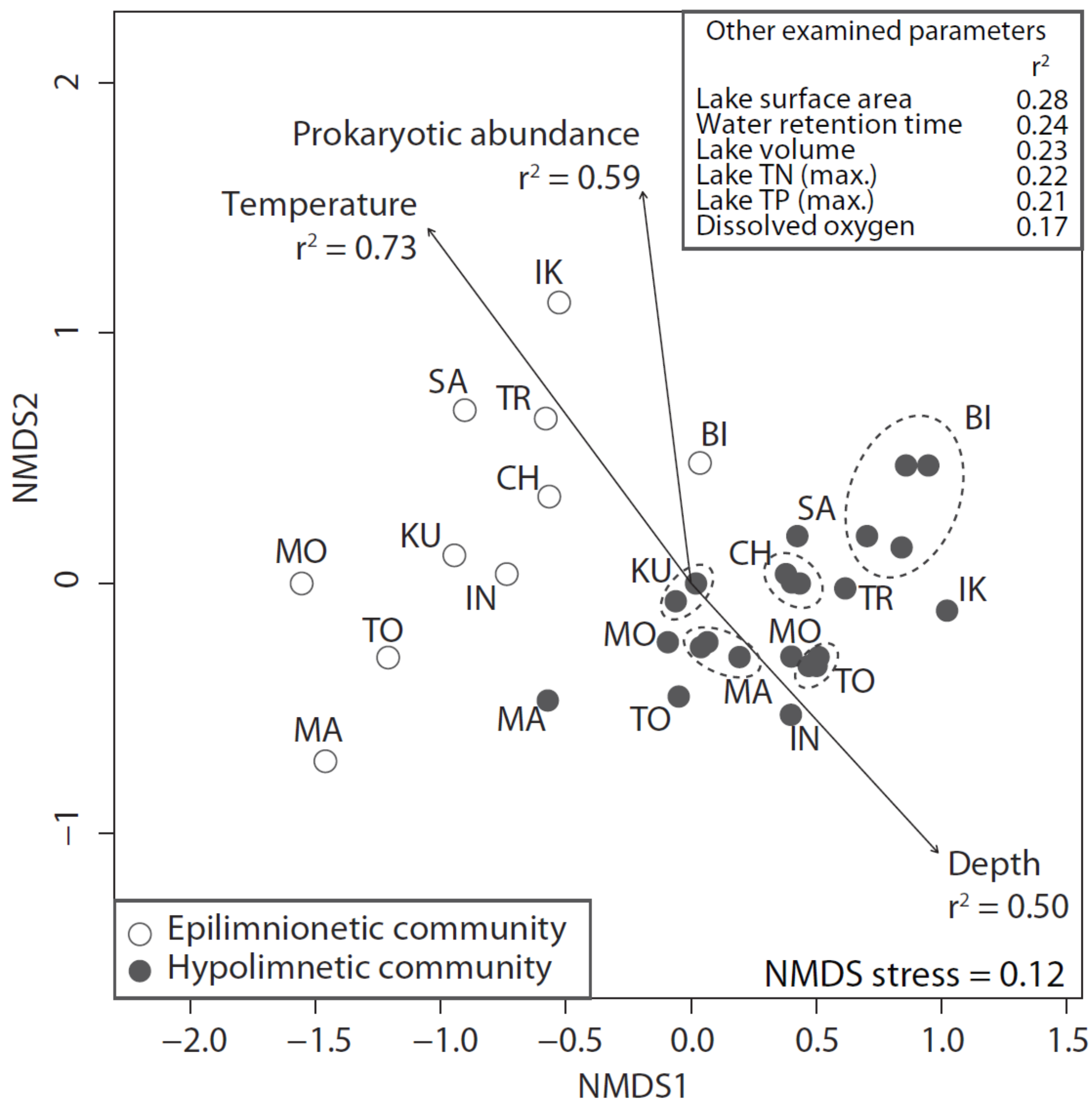
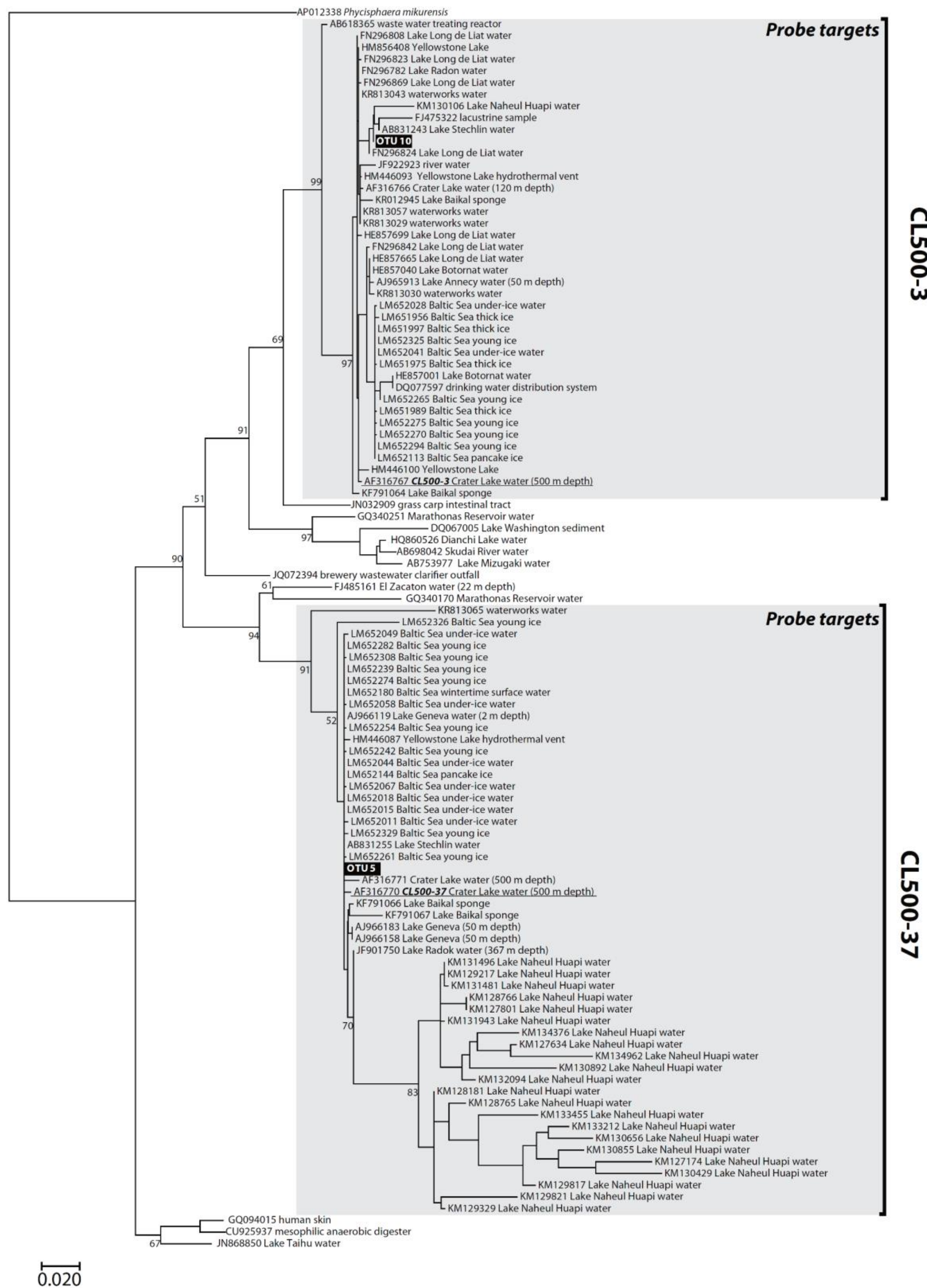


Fig. 4-S3

Non-metric multidimensional scaling (NMDS) ordination of all 33 bacterioplankton communities. The arrows are fitted vectors for the environmental variables calculated using the envfit function. The direction of the arrow indicates the direction at which the gradient of the environmental variable was maximum. The length of the arrow is proportional to the squared correlation coefficient (r^2). Only significant variables ($p < 0.01$; based on 999 permutations) were visualized. The other variables examined are shown in the top right box with the r^2 values.

Fig. 4-S4

Vertical preferences of individual operational taxonomic units (OTUs), mapped by the number of lakes where individual OTUs accounted for >2% (A) and >0.5% (B) of all amplicon reads in each water layer. Data for the hypolimnion were generated by averaging the data at multiple depths in the hypolimnion. The result created by the >1% threshold is shown in Fig. 4-3.



(A)
AP012338 Phycisphaera mikurensis
AB618365 waste water treating reactor
Probe targets
FN296808 Lake Long de Liat water
HM856408 Yellowstone Lake
FN296823 Lake Long de Liat water
FN296782 Lake Radon water
FN296869 Lake Long de Liat water
KR813043 waterworks water
KM130106 Lake Naheul Huapi water
FJ475322 lacustrine sample
AB831243 Lake Stechlin water
OTU 10
FN296824 Lake Long de Liat water
JF922923 river water
HM446093 Yellowstone Lake hydrothermal vent
AF316766 Crater Lake water (120 m depth)
KR012945 Lake Baikal sponge
KR813057 waterworks water
KR813029 waterworks water
HE857699 Lake Long de Liat water
FN296842 Lake Long de Liat water
HE857665 Lake Long de Liat water
HE857040 Lake Botornat water
AJ965913 Lake Annecy water (50 m depth)
KR813030 waterworks water
LM652028 Baltic Sea under-ice water
LM651956 Baltic Sea thick ice
LM651997 Baltic Sea thick ice
LM652325 Baltic Sea young ice
LM652041 Baltic Sea under-ice water
LM651975 Baltic Sea thick ice
HE857001 Lake Botornat water
DQ077597 drinking water distribution system
LM652265 Baltic Sea young ice
LM651989 Baltic Sea thick ice
LM652275 Baltic Sea young ice
LM652270 Baltic Sea young ice
LM652294 Baltic Sea young ice
LM652113 Baltic Sea pancake ice
HM446100 Yellowstone Lake
AF316767 CL500-3 Crater Lake water (500 m depth)
KF791064 Lake Baikal sponge
CL500-3
JN032909 grass carp intestinal tract
GQ340251 Marathonas Reservoir water
DQ067005 Lake Washington sediment
HQ860526 Dianchi Lake water
AB698042 Skudai River water
AB753977 Lake Mizugaki water
JQ072394 brewery wastewater clarifier outfall
FJ485161 El Zacaton water (22 m depth)
GQ340170 Marathonas Reservoir water
KR813065 waterworks water
LM652326 Baltic Sea young ice
Probe targets
LM652049 Baltic Sea under-ice water
LM652282 Baltic Sea young ice
LM652308 Baltic Sea young ice
LM652239 Baltic Sea young ice
LM652274 Baltic Sea young ice
LM652180 Baltic Sea wintertime surface water
LM652058 Baltic Sea under-ice water
AJ966119 Lake Geneva water (2 m depth)
LM652254 Baltic Sea young ice
HM446087 Yellowstone Lake hydrothermal vent
LM652242 Baltic Sea young ice
LM652044 Baltic Sea under-ice water
LM652144 Baltic Sea pancake ice
LM652067 Baltic Sea under-ice water
LM652018 Baltic Sea under-ice water
LM652015 Baltic Sea under-ice water
LM652011 Baltic Sea under-ice water
LM652329 Baltic Sea young ice
AB831255 Lake Stechlin water
LM652261 Baltic Sea young ice
OTU 5
AF316771 Crater Lake water (500 m depth)
AF316770 CL500-37 Crater Lake water (500 m depth)
KF791066 Lake Baikal sponge
KF791067 Lake Baikal sponge
AJ966183 Lake Geneva (50 m depth)
AJ966158 Lake Geneva (50 m depth)
JF901750 Lake Radok water (367 m depth)
KM131496 Lake Naheul Huapi water
KM129217 Lake Naheul Huapi water
KM131481 Lake Naheul Huapi water
KM128766 Lake Naheul Huapi water
KM127801 Lake Naheul Huapi water
KM131943 Lake Naheul Huapi water
KM134376 Lake Naheul Huapi water
KM127634 Lake Naheul Huapi water
KM134962 Lake Naheul Huapi water
KM130892 Lake Naheul Huapi water
KM132094 Lake Naheul Huapi water
KM128181 Lake Naheul Huapi water
KM128765 Lake Naheul Huapi water
KM133455 Lake Naheul Huapi water
KM133212 Lake Naheul Huapi water
KM130656 Lake Naheul Huapi water
KM130855 Lake Naheul Huapi water
KM127174 Lake Naheul Huapi water
KM130429 Lake Naheul Huapi water
KM129817 Lake Naheul Huapi water
KM129821 Lake Naheul Huapi water
KM129329 Lake Naheul Huapi water
CL500-37
GQ094015 human skin
CU925937 mesophilic anaerobic digester
JN868850 Lake Taihu water
99
69
97
91
51
97
90
61
94
91
52
70
83
67
0.020

(B)

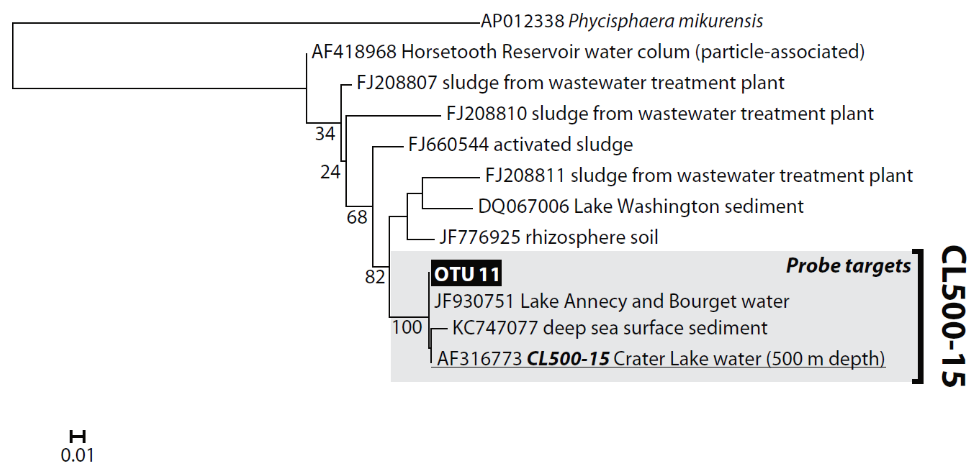


Fig. 4-S5

Maximum likelihood phylogenetic tree illustrating the lineages targeted by the newly constructed fluorescence in situ hybridization (FISH) probes: (A). CL500-3 and CL500-37: (B). CL500-15. All 16S rRNA gene sequences in the database (including partial ones) targeted by the probe were retrieved by the TestProbe tool at the SILVA website and are included in the tree. The neighbor and outgroup sequences were retrieved from the SILVA Ref NR database. The name of the sequence designates the environment where the sequence was retrieved. Sequences targeted by the probe (without mismatch) are shaded. Sequences from which the name of the lineage was derived are underlined. Numbers on the nodes represent bootstrap support from 100 replicates.

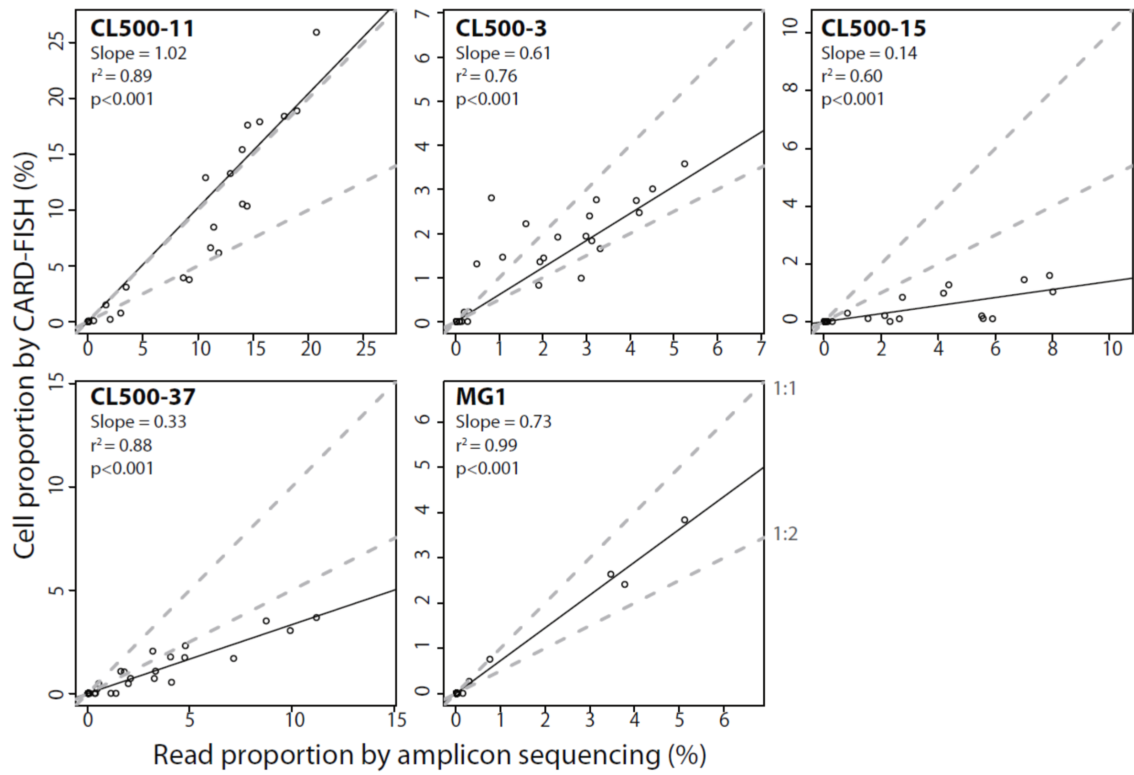


Fig. 4-S6

Pearson's correlation analysis of the relative abundances determined by amplicon sequencing and catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH). Dashed gray lines are drawn to illustrate the 1:1 and 1:2 slopes.

The 10 most abundant OTUs in the hypolimnion of Lake Michigan
(0.22–3 µm fraction; 108 m in September 2013)

Rank	Phylum	OTU name	Read proportion
1	Chloroflexi	CL500-11	26.2%
2	Actinobacteria	acI-B1	11.8%
3	Proteobacteria (α)	LD12	5.7%
4	Proteobacteria (β)	Lhab-A1	3.9%
5	Proteobacteria (β)	LD28	3.4%
6	Bacteroidetes	bacI-A1	2.9%
7	Planctomycetes	plaI-A	2.3%
8	Planctomycetes	CL500-37	1.9%
9	Proteobacteria (β)	Nitrosospira	1.6%
10	Bacteroidetes	bacIII-A	1.6%

The original data were downloaded from the Joint Genome Institute's genome data portal (ID = 1041198).

Fig. 4-S7

The 10 most abundant operational taxonomic units (OTUs) in the 16S rRNA gene amplicon (for the V4 region) sequencing reads taken from the hypolimnion in Lake Michigan (Fujimoto et al., 2016). The 0.22–3 µm fraction data taken at a depth of 108 m at the pelagic station in September (Fa.FL.M110.D.N) was used to represent the hypolimnetic community. Duplicate samples were averaged. The representative sequences of the OTUs and the OTU table were downloaded from the Joint Genome Institute's genome data portal (http://genome.jgi.doe.gov/; Project ID = 1041198) and processed using the same protocol as the present study.

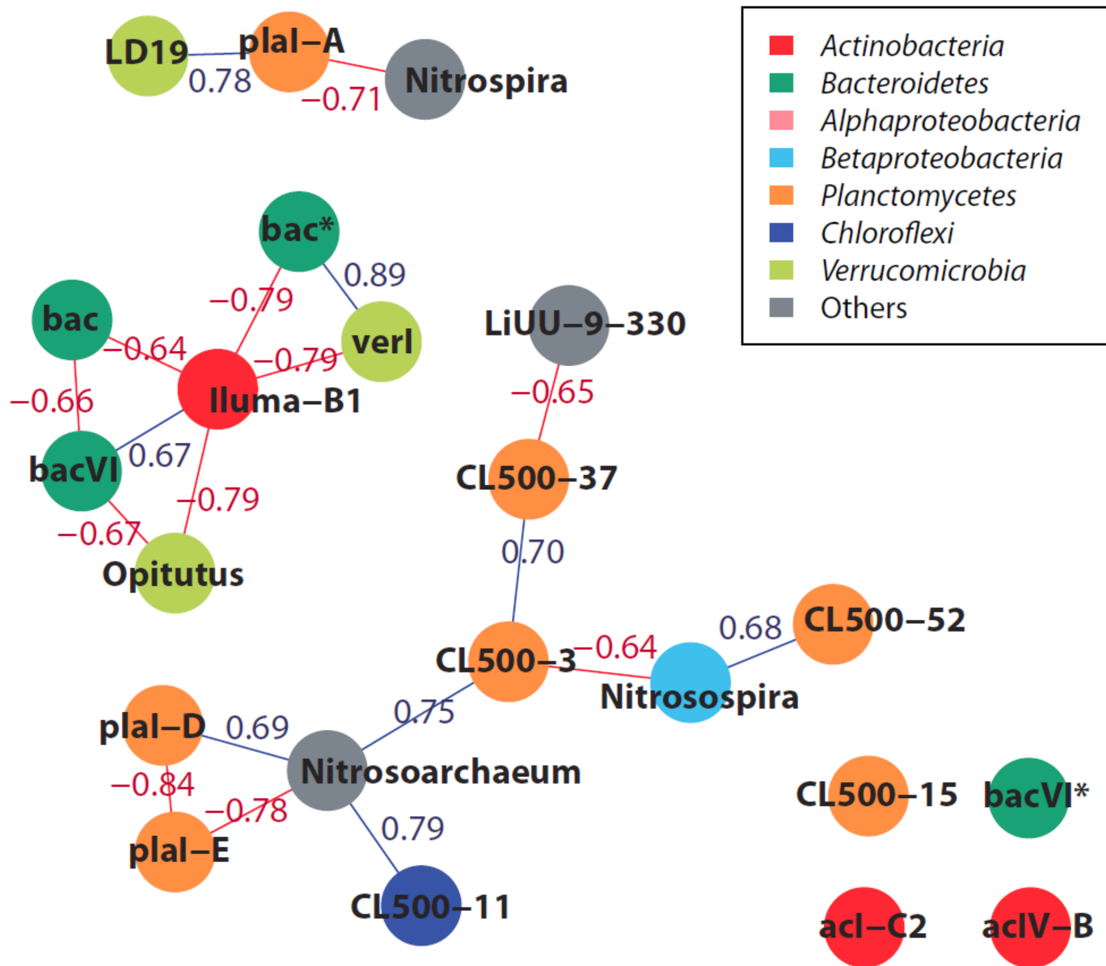


Fig. 4-S8

Spearman's rank coefficient analysis between the 22 hypolimnion inhabitants, evaluated by the averaged hypolimnetic read proportions for each lake. Numbers indicate the correlation coefficient (ρ) and blue and red characters indicate positive and negative correlations, respectively. Only edges with $p < 0.05$ are shown. Asterisks in the group name distinguish the different operational taxonomic units (OTUs) assigned to the same group.

Variation in nucleotide 776 (*E. coli* numbering system) in the CL500–11 16S rRNA sequences.

Region	Country	Reference	Source	
Asia	Japan	This study	The 10 Japanese lakes*	G
	Japan	AB686531	Lake Biwa clone LB65D-54	G
Europe	France	AJ965858	Lake Annecy clone A50Su-16	G
	France	AJ966070	Lake Bourget clone B50Sp-25	G
	France/Switzerland	AJ966121	Lake Geneva clone L2W-58	G
	France	AJ965898	Lake Annecy clone A50W-54	T
	France	AJ965870	Lake Annecy clone A50Su-57	T
	France	AJ965812	Lake Annecy clone A2W-8	T
	France/Switzerland	AJ966228	Lake Geneva clone L50Sp-21	T
	France/Switzerland	AJ966124	Lake Geneva clone L2W-60	T
	France/Switzerland	AJ966120	Lake Geneva clone L2W-57	T
	France/Switzerland	AJ966118	Lake Geneva clone L2W-55	T
	Germany	AB831240	Lake Stechlin clone Initial-OTU7	T
	Germany	AB781443	Lake Stechlin clone NAG-P3-13	T
North America	USA	AF316759	Crater Lake clone CL500-11	T
	USA	Denef et al. (2016)	Metagenome-assembled genome from Lake Michigan	T
	USA	HM446117	Yellowstone Lake clone WT98_73	T
	USA	HM856384	Yellowstone Lake clone YL009	T

* The oligotyping analysis indicated that almost all sequences read in the present study were identical in the 776th position.

Fig. 4-S9

Variation in nucleotide 776 (*E. coli* numbering system; within the V4 region) in the CL500-11 16S rRNA gene sequences.

Supplementary Data 4-1

Composition of the individual oligotypes for each OTU in each sample (i.e., raw data for the oligotyping analysis). The hypolimnion data were generated by averaging the data at multiple depths in the hypolimnion. Asterisks in the group name distinguish the different operational taxonomic units (OTUs) assigned to the same group.

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
acI-B1	GG	Epilimnion	54.8%	60.6%	45.8%	95.0%	86.5%	98.0%	55.8%	99.8%	93.2%	98.9%
		Hypolimnion	55.6%	35.4%	61.7%	77.1%	48.1%	53.4%	41.9%	99.5%	44.6%	33.9%
	AG	Epilimnion	45.2%	18.8%	54.2%	0.0%	12.9%	2.0%	44.2%	0.1%	0.7%	0.3%
		Hypolimnion	44.2%	11.1%	38.3%	0.0%	27.6%	7.1%	17.1%	0.0%	4.1%	7.1%
	GA	Epilimnion	0.0%	20.6%	0.0%	5.0%	0.6%	0.0%	0.0%	0.1%	6.2%	0.8%
		Hypolimnion	0.2%	53.5%	0.0%	22.9%	24.4%	39.5%	41.0%	0.5%	51.3%	58.9%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
acI-A7	AGAUG	Epilimnion	0.0%	47.9%	3.5%	58.4%	63.8%	26.7%	71.6%	12.0%	81.2%	29.2%
		Hypolimnion	4.3%	70.3%	1.9%	53.9%	42.2%	66.3%	49.2%	73.1%	55.7%	57.0%
	AGAUA	Epilimnion	21.3%	0.0%	14.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.4%	0.0%
		Hypolimnion	90.4%	23.0%	95.1%	0.3%	48.9%	4.7%	19.9%	0.6%	10.8%	15.2%
	CGAUG	Epilimnion	78.8%	51.8%	82.5%	31.2%	36.2%	73.3%	28.4%	88.0%	12.1%	66.7%
		Hypolimnion	5.3%	5.4%	3.0%	10.9%	1.2%	3.3%	3.9%	3.3%	4.7%	0.0%
	AUAUA	Epilimnion	0.0%	0.4%	0.0%	10.4%	0.0%	0.0%	0.0%	0.0%	0.0%	4.2%
		Hypolimnion	0.0%	1.2%	0.0%	34.9%	0.5%	11.1%	15.9%	23.0%	1.7%	22.8%
	AUAGA	Epilimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
		Hypolimnion	0.0%	0.0%	0.0%	0.0%	7.3%	14.4%	11.0%	0.0%	0.0%	5.1%
	AUGUA	Epilimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.4%	0.0%
		Hypolimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%	0.0%	0.0%	27.1%	0.0%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
Lhab-A1	AAUU-	Epilimnion	40.9%	73.8%	60.9%	60.1%	73.2%	97.9%	36.9%	88.9%	45.5%	81.8%
		Hypolimnion	28.8%	23.6%	13.6%	8.7%	32.0%	16.5%	18.0%	15.8%	16.8%	13.8%
	GCUU-	Epilimnion	32.1%	15.4%	27.2%	32.5%	23.2%	0.7%	18.8%	8.5%	48.7%	0.0%
		Hypolimnion	10.4%	32.4%	2.4%	71.8%	46.2%	75.4%	70.2%	56.1%	45.9%	82.8%
	ACUU-	Epilimnion	2.9%	3.8%	2.7%	1.2%	0.0%	0.4%	0.0%	0.0%	1.3%	4.5%
		Hypolimnion	52.7%	37.6%	70.2%	3.9%	16.3%	6.0%	0.5%	2.9%	5.4%	0.0%
	GCUUG	Epilimnion	13.4%	1.5%	0.6%	1.8%	0.0%	0.0%	5.6%	0.0%	0.6%	0.0%
		Hypolimnion	0.6%	2.2%	0.3%	5.8%	1.4%	0.4%	6.1%	18.0%	4.2%	0.0%
	AAUUG	Epilimnion	9.1%	4.6%	2.3%	1.8%	2.8%	0.0%	11.9%	2.0%	3.2%	13.6%
		Hypolimnion	1.3%	0.9%	0.8%	0.0%	1.6%	0.0%	4.8%	4.3%	1.9%	0.0%
	GCAU-	Epilimnion	0.0%	0.0%	0.0%	1.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
		Hypolimnion	0.0%	2.3%	1.2%	7.8%	2.2%	0.4%	0.0%	0.7%	24.9%	0.0%
	ACUUG	Epilimnion	1.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
		Hypolimnion	6.1%	0.9%	11.1%	1.0%	0.3%	0.0%	0.0%	0.7%	0.0%	0.0%
	GAUU-	Epilimnion	0.2%	0.8%	5.8%	1.2%	0.7%	1.1%	4.1%	0.7%	0.6%	0.0%
		Hypolimnion	0.2%	0.0%	0.3%	1.0%	0.0%	1.2%	0.4%	1.4%	0.8%	3.4%
	AAUC-	Epilimnion	0.0%	0.0%	0.6%	0.0%	0.0%	0.0%	17.8%	0.0%	0.0%	0.0%
		Hypolimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	AAUCG	Epilimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	5.0%	0.0%	0.0%	0.0%
		Hypolimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
bacI-A1	**ACG**	Epilimnion	0.0%	27.7%	5.9%	16.1%	13.5%	0.0%	0.0%	5.4%	50.4%	26.1%
		Hypolimnion	48.2%	29.3%	67.8%	23.4%	60.8%	65.3%	59.0%	37.7%	69.2%	84.2%
	GCG	Epilimnion	100.0%	64.9%	94.1%	63.1%	47.2%	0.0%	18.2%	12.2%	41.8%	39.1%
		Hypolimnion	51.3%	68.5%	32.1%	76.6%	30.8%	34.7%	36.4%	61.4%	30.6%	8.8%
	ACA	Epilimnion	0.0%	2.1%	0.0%	16.5%	9.3%	68.1%	81.8%	43.8%	4.7%	4.3%
		Hypolimnion	0.3%	0.6%	0.2%	0.0%	0.0%	0.0%	1.1%	0.8%	0.0%	7.0%
	GUA	Epilimnion	0.0%	0.0%	0.0%	3.6%	16.1%	31.9%	0.0%	38.1%	0.3%	30.4%
		Hypolimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%
	GCA	Epilimnion	0.0%	5.3%	0.0%	0.8%	14.0%	0.0%	0.0%	0.5%	2.7%	0.0%
		Hypolimnion	0.2%	1.6%	0.0%	0.0%	8.4%	0.0%	3.5%	0.0%	0.3%	0.0%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
Iluma-A1	**AGUC**	Epilimnion	100.0%	100.0%	100.0%	80.6%	14.6%	0.0%	NA	27.6%	39.8%	0.0%
		Hypolimnion	79.6%	91.0%	46.0%	69.7%	66.2%	53.3%	80.7%	51.2%	80.0%	79.9%
	GGUG	Epilimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	NA	0.0%	2.3%	0.0%
		Hypolimnion	20.2%	5.3%	32.4%	0.0%	19.4%	28.6%	19.0%	0.0%	10.7%	16.8%
	GAUC	Epilimnion	0.0%	0.0%	0.0%	0.0%	84.1%	99.4%	NA	0.0%	13.1%	1.0%
		Hypolimnion	0.0%	2.8%	0.0%	0.0%	10.7%	16.2%	0.0%	0.0%	6.3%	0.7%
	GGCG	Epilimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	NA	72.4%	41.5%	99.0%
		Hypolimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	32.6%	1.7%	2.7%
	AGAC	Epilimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	NA	0.0%	0.0%	0.0%
		Hypolimnion	0.1%	0.0%	21.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	GGUC	Epilimnion	0.0%	0.0%	0.0%	19.4%	0.0%	0.6%	NA	0.0%	0.6%	0.0%
		Hypolimnion	0.1%	0.8%	0.5%	30.3%	1.7%	1.9%	0.3%	16.3%	1.3%	0.0%
	GGAC	Epilimnion	0.0%	0.0%	0.0%	0.0%	1.3%	0.0%	NA	0.0%	2.8%	0.0%
		Hypolimnion	0.0%	0.0%	0.0%	0.0%	2.1%	0.0%	0.0%	0.0%	0.0%	0.0%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
Iluma-A2	**G**	Epilimnion	NA	100.0%	NA	NA	9.3%	0.0%	NA	9.2%	32.3%	NA
		Hypolimnion	99.7%	99.6%	100.0%	100.0%	96.3%	99.4%	100.0%	95.1%	99.1%	100.0%
	C	Epilimnion	NA	0.0%	NA	NA	75.7%	87.3%	NA	59.0%	45.5%	NA
		Hypolimnion	0.3%	0.0%	0.0%	0.0%	0.0%	0.6%	0.0%	3.1%	0.5%	0.0%
	A	Epilimnion	NA	0.0%	NA	NA	15.0%	12.7%	NA	31.8%	22.2%	NA
		Hypolimnion	0.0%	0.4%	0.0%	0.0%	3.7%	0.0%	0.0%	1.8%	0.5%	0.0%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
LD28	**--**	Epilimnion	NA	60.0%	NA	68.8%	16.7%	NA	NA	53.2%	50.0%	45.5%
		Hypolimnion	57.2%	59.7%	46.3%	41.7%	45.7%	47.9%	67.6%	84.1%	60.6%	40.5%
	-G	Epilimnion	NA	15.0%	NA	12.5%	16.7%	NA	NA	22.6%	11.9%	18.2%
		Hypolimnion	14.4%	20.1%	36.4%	25.9%	28.9%	51.3%	19.5%	5.8%	11.2%	21.4%
	G-	Epilimnion	NA	25.0%	NA	18.8%	66.7%	NA	NA	24.2%	38.1%	33.3%
		Hypolimnion	26.7%	18.5%	16.2%	26.9%	24.9%	0.8%	12.9%	8.7%	26.5%	35.7%
	GG	Epilimnion	NA	0.0%	NA	0.0%	0.0%	NA	NA	0.0%	0.0%	3.0%
		Hypolimnion	1.7%	1.7%	1.1%	5.6%	0.6%	0.0%	0.0%	1.4%	1.7%	2.4%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
CL500-3	**C**	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	93.1%	93.5%	92.2%	92.8%	NA	84.2%	74.4%	89.5%	91.3%	92.2%
	-	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	6.9%	6.5%	7.8%	7.2%	NA	15.8%	25.6%	10.5%	8.7%	7.8%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
CL500-11	U	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	100.0%	NA
		Hypolimnion	98.5%	100.0%	95.4%	NA	94.2%	59.0%	96.4%	NA	98.2%	97.9%
	A	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	0.0%	NA
		Hypolimnion	1.5%	0.0%	4.6%	NA	5.8%	41.0%	3.6%	NA	1.8%	2.1%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
CL500-37	-	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	99.3%	100.0%	99.1%	98.8%	98.7%	100.0%	NA	NA	99.2%	97.3%
	A	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	0.7%	0.0%	0.9%	1.2%	1.3%	0.0%	NA	NA	0.8%	2.7%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
acIV-B	G	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	90.0%	NA
		Hypolimnion	96.9%	100.0%	98.8%	NA	98.0%	93.8%	99.4%	96.6%	95.1%	94.4%
	-	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	10.0%	NA
		Hypolimnion	3.1%	0.0%	1.2%	NA	2.0%	6.3%	0.6%	3.4%	4.9%	5.6%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
CL500-15	A	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	56.2%	NA	66.3%	NA	64.6%	73.1%	71.4%	NA	62.9%	NA
	-	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	43.8%	NA	33.7%	NA	35.4%	26.9%	28.6%	NA	37.1%	NA

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
plaI-A	GA	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	73.7%	NA
		Hypolimnion	100.0%	100.0%	100.0%	95.0%	100.0%	96.3%	74.5%	58.8%	70.4%	100.0%
	AG	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	26.3%	NA
		Hypolimnion	0.0%	0.0%	0.0%	5.0%	0.0%	3.7%	0.0%	41.2%	29.6%	0.0%
	GG	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	0.0%	NA
		Hypolimnion	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	25.5%	0.0%	0.0%	0.0%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
bacVI*	GA	Epilimnion	NA	3.4%	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	100.0%	NA	100.0%	0.8%	100.0%	100.0%	NA	NA	98.8%	100.0%
	AA	Epilimnion	NA	0.0%	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	0.0%	NA	0.0%	99.2%	0.0%	0.0%	NA	NA	0.0%	0.0%
	GG	Epilimnion	NA	96.6%	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	0.0%	NA	0.0%	0.0%	0.0%	0.0%	NA	NA	1.3%	0.0%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
LD19	C	Epilimnion	NA	NA	97.4%	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	98.0%	96.4%	98.1%	100.0%	100.0%	100.0%	97.4%	NA	NA	NA
	G	Epilimnion	NA	NA	2.6%	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	2.0%	3.6%	1.9%	0.0%	0.0%	0.0%	2.6%	NA	NA	NA

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
CL500-52	-	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	92.9%	NA	NA	90.0%	81.1%	NA
	A	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	7.1%	NA	NA	10.0%	18.9%	NA

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
Nitrospira	A	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	92.3%	100.0%	100.0%	NA	NA	56.3%	51.7%	81.3%
	G	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	7.7%	0.0%	0.0%	NA	NA	43.8%	48.3%	18.8%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
Nitrosoarchaeum	AGCA	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	NA	NA	NA	88.8%	28.4%
	AGAG	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	NA	NA	NA	0.0%	37.0%
	UGCA	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	NA	NA	NA	10.6%	2.5%
	AGAA	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	NA	NA	NA	0.6%	14.8%
	AACA	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	NA	NA	NA	0.0%	17.3%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
Nitrosospira	GU	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	73.2%	0.0%	0.0%	76.2%	NA	NA
	CU	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	13.5%	0.0%	0.0%	7.1%	NA	NA
	CC	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	0.0%	89.5%	100.0%	11.9%	NA	NA
	GG	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	13.3%	10.5%	0.0%	4.8%	NA	NA

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
plal-D	A	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	NA	NA	NA	100.0%	0.0%
	G	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	NA	NA	NA	0.0%	100.0%

	oligotype		MA	KU	TO	IN	CH	SA	MO	TR	BI	IK
bac*	-U	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	92.9%	NA	91.9%	59.6%	NA
	AU	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	0.0%	NA	0.0%	22.6%	NA
	A-	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	0.0%	NA	0.0%	13.2%	NA
	--	Epilimnion	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		Hypolimnion	NA	NA	NA	NA	NA	7.1%	NA	8.1%	4.7%	NA

Supplementary Data 4-2

Proportion of CARD-FISH to DAPI positive cells in each sample (i.e., raw data for Fig. 4-6).

CL500-11

(m)	Mashu	(m)	Kusharo	(m)	Toya	(m)	Inawashiro	(m)	Chuzenji	(m)	Sai	(m)	Motosu	(m)	T-Reservoir	(m)	Biwa	(m)	Ikeda
5	0.0%	1.5	0.0%	5	0.0%	5	0.0%	5	0.0%	10	0.0%	10	0.0%	5	0.0%	2	0.8%	5	0.0%
10	0.0%	10	0.0%	10	0.0%	20	0.0%	10	0.0%	30	0.3%	20	0.0%	60	0.0%	40	18.4%	70	18.9%
20	0.0%	20	0.1%	20	0.0%	80	0.0%	20	0.2%	50	0.2%	50	3.1%	120	0.0%	60	25.9%		
30	0.1%	30	0.2%	30	0.4%			50	13.3%	70	0.0%	100	6.6%			80	17.9%		
50	1.5%	50	0.1%	50	6.1%			100	15.4%							95	17.6%		
100	3.9%	110	0.1%	100	10.3%			120	12.9%										
200	3.7%			150	10.5%														
				165	8.4%														

CL500-3

(m)	Mashu	(m)	Kusharo	(m)	Toya	(m)	Inawashiro	(m)	Chuzenji	(m)	Sai	(m)	Motosu	(m)	T-Reservoir	(m)	Biwa	(m)	Ikeda
5	0.0%	1.5	0.0%	5	0.0%	5	0.0%	5	0.0%	10	0.0%	10	0.0%	5	0.0%	2	0.0%	5	0.0%
10	0.0%	10	0.0%	10	0.0%	20	0.0%	10	0.0%	30	0.5%	20	0.0%	60	1.5%	40	1.9%	70	1.0%
20	0.0%	20	0.0%	20	0.0%	80	1.4%	20	0.0%	50	1.3%	50	2.8%	120	0.0%	60	2.7%		
30	0.0%	30	2.3%	30	0.0%			50	0.0%	70	0.0%	100	2.2%			80	3.0%		
50	0.0%	50	1.8%	50	2.4%			100	0.2%							95	3.6%		
100	1.4%	110	0.8%	100	2.5%			120	0.2%										
200	1.9%			150	2.8%														
				165	1.7%														

CL500-37

(m)	Mashu	(m)	Kusharo	(m)	Toya	(m)	Inawashiro	(m)	Chuzenji	(m)	Sai	(m)	Motosu	(m)	T-Reservoir	(m)	Biwa	(m)	Ikeda
5	0.0%	1.5	0.0%	5	0.0%	5	0.0%	5	0.0%	10	0.0%	10	0.0%	5	0.0%	2	0.0%	5	0.0%
10	0.0%	10	0.0%	10	0.0%	20	0.0%	10	0.0%	30	0.9%	20	0.0%	60	0.0%	40	2.0%	70	0.7%
20	0.0%	20	0.0%	20	0.0%	80	3.7%	20	0.0%	50	0.5%	50	0.0%	120	0.0%	60	0.5%		
30	0.0%	30	0.7%	30	0.0%			50	0.0%	70	0.0%	100	0.2%			80	0.7%		
50	0.0%	50	1.0%	50	2.3%			100	0.5%							95	1.1%		
100	1.8%	110	1.1%	100	3.0%			120	0.0%										
200	1.7%			150	1.7%														
				165	3.5%														

CL500-15

(m)	Mashu	(m)	Kusharo	(m)	Toya	(m)	Inawashiro	(m)	Chuzenji	(m)	Sai	(m)	Motosu	(m)	T-Reservoir	(m)	Biwa	(m)	Ikeda
5	0.0%	1.5	0.0%	5	0.0%	5	0.0%	5	0.0%	10	0.0%	10	0.0%	5	0.0%	2	0.0%	5	0.0%
10	0.0%	10	0.0%	10	0.0%	20	0.0%	10	0.0%	30	0.2%	20	0.0%	60	0.0%	40	1.0%	70	0.0%
20	0.0%	20	0.0%	20	0.0%	80	0.0%	20	0.0%	50	1.6%	50	0.0%	120	0.0%	60	1.3%		
30	0.0%	30	0.0%	30	0.0%			50	0.1%	70	0.0%	100	0.1%			80	1.4%		
50	0.0%	50	0.0%	50	0.0%			100	0.2%							95	1.0%		
100	0.3%	110	0.0%	100	0.0%			120	0.1%										
200	0.1%			150	0.2%														
				165	0.8%														

MG1

(m)	Mashu	(m)	Kusharo	(m)	Toya	(m)	Inawashiro	(m)	Chuzenji	(m)	Sai	(m)	Motosu	(m)	T-Reservoir	(m)	Biwa	(m)	Ikeda
5	0.0%	1.5	0.0%	5	0.0%	5	0.0%	5	0.0%	10	0.0%	10	0.0%	5	0.0%	2	0.0%	5	0.0%
10	0.0%	10	0.0%	10	0.0%	20	0.0%	10	0.0%	30	0.0%	20	0.0%	60	0.0%	40	2.6%	70	3.8%
20	0.0%	20	0.0%	20	0.0%	80	0.0%	20	0.0%	50	0.0%	50	0.0%	120	0.0%	60	2.4%		
30	0.0%	30	0.0%	30	0.0%			50	0.0%	70	0.0%	100	0.0%			80	0.8%		
50	0.0%	50	0.0%	50	0.0%			100	0.0%							95	0.3%		
100	0.0%	110	0.0%	100	0.0%			120	0.0%										
200	0.0%			150	0.0%														
				165	0.0%														

Sum of the five lineages

(m)	Mashu	(m)	Kusharo	(m)	Toya	(m)	Inawashiro	(m)	Chuzenji	(m)	Sai	(m)	Motosu	(m)	T-Reservoir	(m)	Biwa	(m)	Ikeda
5	0.0%	1.5	0.0%	5	0.0%	5	0.0%	5	0.0%	10	0.0%	10	0.0%	5	0.0%	2	0.8%	5	0.0%
10	0.0%	10	0.0%	10	0.0%	20	0.0%	10	0.0%	30	1.9%	20	0.0%	60	1.5%	40	26.0%	70	24.4%
20	0.0%	20	0.1%	20	0.0%	80	5.0%	20	0.2%	50	3.6%	50	5.9%	120	0.0%	60	32.9%		
30	0.1%	30	3.2%	30	0.4%			50	13.3%	70	0.0%	100	9.1%			80	23.8%		
50	1.5%	50	2.9%	50	10.8%			100	16.3%							95	23.5%		
100	7.4%	110	1.9%	100	15.8%			120	13.2%										
200	7.5%			150	15.2%														
				165	14.4%														

Chapter 5: General discussion

Importance and achievements of the present study

In this thesis, I aimed to establish a general overview of bacterioplankton lineages (defined by >97% identity in the 16S rRNA gene sequence) inhabiting the oxygenated hypolimnion of deep freshwater lakes. First, the quantitative survey of the horizontal, vertical, and seasonal dynamics of CL500-11 in Lake Biwa indicated the existence of a hypolimnion-specific bacterioplankton lineage (Chapter 2). Second, the analysis of spatiotemporal partitioning of bacterioplankton assemblages in Lake Biwa revealed that the community was significantly different in the epilimnion and hypolimnion at the level of phylum composition. The analysis identified several hypolimnion-specific lineages mainly affiliated with the phyla *Chloroflexi*, *Planctomycetes*, and *Thaumarchaeota*, which had not been characterized as typical bacterioplankton in surface freshwaters (Zwart et al., 2002; Newton et al., 2011) (Chapter 3). Finally, the predominance of the hypolimnion-specific lineages was verified in 10 deep freshwater lakes across Japan using both comprehensive (amplicon sequencing) and quantitative (FISH) methods. An analysis of single-nucleotide variation of the amplicons (oligotyping) further suggested the presence of different subpopulations between lakes and water layers among the lineages occurring in the entire water column (e.g., acI-B1 and acI-A7) (Chapter 4).

In summary, the data in this thesis revealed the existence of hypolimnion-specific bacterioplankton that are ubiquitous and quantitatively significant in multiple deep freshwater lakes and provided the first comprehensive information on their distribution and abundance. These results indicate that the ecosystem and biogeochemical cycling in the oxygenated hypolimnion are driven by the specific members of bacterioplankton that are distinct from those in the epilimnion.

Limitation of the studies that need to be addressed in the future work

Although this study sampled as much as 10 deep freshwater lakes, it did not cover the

all types of lakes; lakes with >250 m depths and on other continents were not investigated. Indeed, several lineages reported in the oxygenated hypolimnia of other lakes were not detected in the present study, as discussed in Chapter 4. The data in Chapter 4 also indicated that the hypolimnetic bacterial community was different between the investigated lakes (Fig. 4-4). These results imply that each hypolimnion-specific member adapts to different environment in oxygenated hypolimnia. Unfortunately, the 10 lakes' data were not enough to determine factors affecting the distribution patterns of the individual hypolimnion-specific lineages (Fig. 4-4). Further information from more lakes would allow to reach more general and robust conclusion. Lake Tazawa (maximum depth = 423 m), Lake Shikotsu (360 m), and Lake Towada (326 m) are the possible ultra-deep lakes that would be sampled in the future works. To collect data from lakes on other continents, I have been conducting FISH and amplicon sequencing analyses in seven deep European freshwater lakes with oxygenated hypolimnion (unpublished data). Since the oligotyping analysis suggested that a partial sequence of the 16S rRNA gene is not informative enough to observe ecologically meaningful taxonomic units (Fig. 4-5), increasing phylogenetic resolution by sequencing and comparing the full-length 16S rRNA gene, the whole rrn operon, or even full genome of the hypolimnion-specific lineages should be the focus of future studies. These additional investigations will endorse, expand, or modify the conclusion of the present study for more comprehensive, widely-accepted consensus of the bacterioplankton community inhabiting oxygenated hypolimnion.

In the present study, the ecophysiologies of individual hypolimnion-specific lineages have been hypothesized using available information such as their abundance, distribution pattern, and characteristics of closely related strains (Chapter 2, 3, and 4). In order to directly examine their ecophysiology, cultivation of the isolated strains is an absolute goal. However, it is a challenging task because most of the environmental bacteria, in particular ones occurring in oligotrophic environments, are difficult to be cultivated (Puspita et al., 2012). Indeed, all of my attempt to isolate the hypolimnion-specialists have not yet been successful. For cultivation-independent characterization,

direct genome sequencing of environmental bacteria (i.e., single-cell genomics and metagenomics) is now becoming a common approach, which gives estimation for the metabolic capabilities of respective lineages based on their assembled genomes (Parks et al., 2017; Woyke et al., 2017). In my preliminarily research in Lake Biwa, high-quality metagenome-assembled genomes of several predominant hypolimnion-specialists, including CL500-11, CL500-15, *Nitrospira*, *Nitrosospira*, and *Ca.* Nitrosoarchaeum, have been successfully assembled (unpublished data). In addition to the metagenomics, a metatranscriptomic analysis will be carried out to acquire the gene expression patterns of individual lineages over depths and seasons. Combined with previously reported physicochemical and biological properties of the lakes, the results from these meta-omics analyses will allow to reach a firmer understanding of the ecological and biogeochemical processes driven by the hypolimnion-specific bacterioplankton lineages.

Further possibilities and long-term perspectives of the study

Not only to understand the ecosystem and biogeochemical processes in deep freshwater lakes, the study could be of more impact if it can be further applied to broader environments. Here, I propose that the microbial ecology in deep freshwater lakes has a potential to be expanded to answer more general scientific questions in a long-term perspective. The deep freshwater microbial ecosystems show much more even, stable, and predictable dynamics (Figs. 2-4 and 3-4), compared to more disturbance-prone, heterogenic systems such as soils and eutrophic waters. Samples from oligo-mesotrophic deep freshwater lakes contain less non-bacterial particles, so that microscopy (Figs. 2-3 and 4-6), size-fractionation, and DNA extraction are more feasible compared with more particle-rich samples from soil, sediment, and eutrophic systems. In contrast to marine studies, seasonal and vertical sampling is significantly easier and less expensive. Since individual lakes have different physicochemical, biological, and geological properties, comparison or reproduction of a result in different ecosystems can be easily performed by sampling multiple lakes. By making the best use of these technical advantages, I consider that the microbial ecology in deep freshwater lakes could be developed to

answer questions of broader scientific disciplines, including general microbiology, general ecology, and even general life sciences (Fig. 5-1). For example, the fact that the sequence of the V4 and V5 regions of the16S rRNA gene of CL500-11 had a conserved single-nucleotide divergence between continents but was completely identical across Japan (Fig. 4-S9), suggests that the CL500-11 lineage is a good case to study the phylogeographic and evolutional background of a bacterial lineage occurring geologically isolated habitats. Viral metagenomics, which is revealing the earth's vast viral diversity and ecology led by the recent development of sequencing and bioinformatics technologies (Paez-Espino et al., 2016; Roux et al., 2016), is also worth to be performed in the deep freshwater ecosystems regarding the uniqueness of the bacterial community. The long-stable hypolimnetic microbial community during the stratified period followed by the rapid corruption of the population during the mixing period (Figs. 2-4, 3-2 and 3-4) imply that there could be unknown dynamic viral-host interactions. These subjects and several other topics shown in Fig. 5-1 are already ongoing for preliminary analyses. In my future works, I will, not only understand the microbial ecosystem in deep freshwater lakes, but also interdisciplinary expand the range of my interest and techniques to make the best use of the future possibility of studying this intriguing but largely unexplored ecosystem.

Tables and Figures

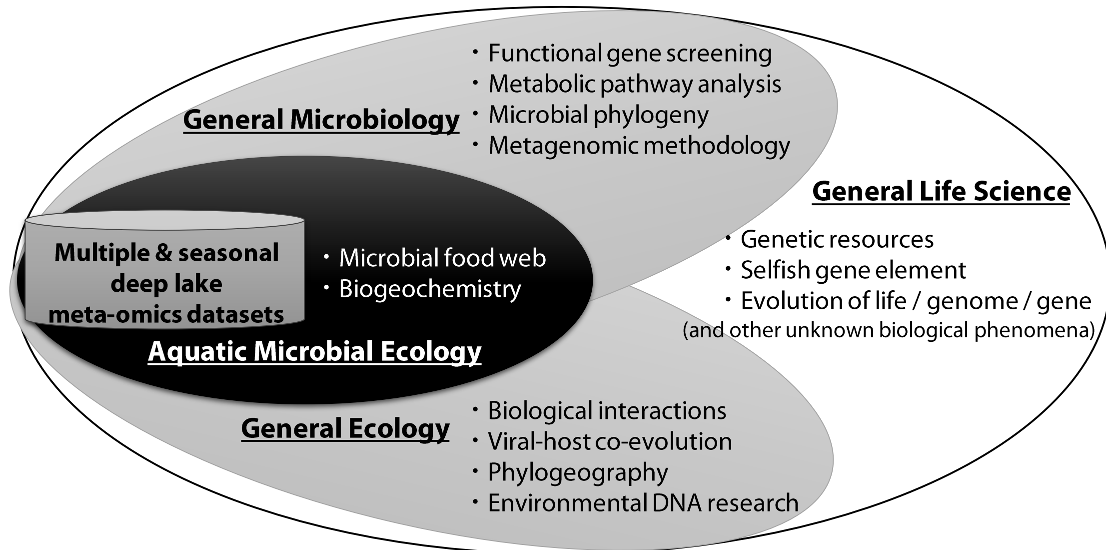


Figure 5-1

Overview of the long-term perspective of the study. The meta-omics datasets generated from deep lakes' microbial ecology could possibly be applied not only to aquatic microbial ecology, but also to questions of general microbiology, general ecology, or even general life science.

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