

Flavobacteria Blooms in Four Eutrophic Lakes: Linking Population Dynamics of Freshwater Bacterioplankton to Resource Availability^{∇†}

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Received 31 October 2006/Accepted 2 April 2007

Heterotrophic bacteria are major contributors to biogeochemical cycles and influence water quality. Still, the lack of representative isolates and the few quantitative surveys leave the ecological role and significance of single bacterial populations to be revealed. Here we analyzed the diversity and dynamics of freshwater *Flavobacteria* populations in four eutrophic temperate lakes. From each lake, clone libraries were constructed using primers specific for either the class *Flavobacteria* or *Bacteria*. Sequencing of 194 *Flavobacteria* clones from 8 libraries revealed a diverse freshwater *Flavobacteria* community and distinct differences among lakes. Abundance and seasonal dynamics of *Flavobacteria* were assessed by quantitative PCR with class-specific primers. In parallel, the dynamics of individual populations within the *Flavobacteria* community were assessed with terminal restriction fragment length polymorphism analysis using identical primers. The contribution of *Flavobacteria* to the total bacterioplankton community ranged from 0.4 to almost 100% (average, 24%). Blooms where *Flavobacteria* represented more than 30% of the bacterioplankton were observed at different times in the four lakes. In general, high proportions of *Flavobacteria* appeared during episodes of high bacterial production. Phylogenetic analyses combined with *Flavobacteria* community fingerprints suggested dominance of two *Flavobacteria* lineages. Both drastic alterations in total *Flavobacteria* and in community composition of this class significantly correlated with bacterial production, emphasizing that resource availability is an important driver of heterotrophic bacterial succession in eutrophic lakes.

Over the last decades, information has accumulated regarding seasonal succession of phytoplankton in lakes. In general, diatoms dominate spring blooms in most eutrophic lakes, and massive developments of cyanobacteria (i.e., cyanobacterial blooms) are characteristic for late-summer conditions (17). On the contrary, our understanding of bacterioplankton dynamics in freshwaters is scarce, even if these mostly organoheterotrophic microorganisms are major contributors to processes that control water quality and the fate of pollutants. Recent studies have identified many uncultured and apparently widely distributed bacterial groups in freshwaters (see, for example, references 8, 13, and 46), but still little is known about the seasonality of bacterial taxa in such systems.

Three phyla, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*, usually occur at high frequencies in clone libraries from lakes (for example, see references 8, 13, 22, and 46). Quantitative studies based on fluorescence in situ hybridization have revealed that members of the *Bacteroidetes* and *Proteobacteria* exhibit high temporal and spatial variation (10, 12, 19, 33, 34). It has been suggested that the ability of *Bacteroidetes* and *Proteobacteria* to rapidly exploit bioavailable organic matter and colonize aggregates could be responsible for their dynamic distribution patterns (1, 28, 44). Understanding of the role of bacterial populations relies on studies that simultaneously de-

termine the distribution of single bacterial populations and properties of the environment. Only a few studies have provided first explanations for population patterns on spatial and temporal scales (see, for example, references 5, 18, and 23–25). For example, two studies based on PCR have shown that freshwater phytoplankton blooms foster bacterial communities where members of the class *Flavobacteria* are well represented (8, 20). Even if *Flavobacteria* are frequently observed in freshwater clone libraries (8, 13, 46), there are only a few freshwater systems where the distribution of groups within the *Bacteroidetes* has been assessed quantitatively (12, 24, 33, 34).

The present study reveals seasonal distribution patterns for the class *Flavobacteria* in four eutrophic lakes situated in the temperate zone of central Sweden. Culture-independent techniques (quantitative PCR [Q-PCR], terminal restriction fragment length polymorphism [T-RFLP], cloning, and sequence analysis) were applied in parallel to study the diversity and dynamics of *Flavobacteria* at the class and subclass levels. Dynamics of *Flavobacteria* communities were also compared to environmental-state variables to identify factors regulating this abundant but largely uncultured group of freshwater bacteria and elucidate their ecological roles in freshwater ecosystems.

MATERIALS AND METHODS

Water samples were collected from four lakes located in the same geographical region of central Sweden. Lake characteristics have been described in detail elsewhere (8). The four lakes differ in the types of *Cyanobacteria* that usually dominate their late-summer cyanobacterial blooms. *Microcystis aeruginosa* dominated in Lake Ekoln and *Gloeotrichia echinulata* dominated in Lake Erken, whereas multiple cyanobacterial species, such as the *Anabaena* and *Aphanizomenon* spp., dominated in the shallow lakes Limmaren and Vallentunasjön.

Sampling. The four lakes were sampled once in 2002 and seven times between May and October in 2003. Single surface water samples (0 to 0.5 m) were

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

∇ Published ahead of print on 13 April 2007.

collected from each lake using acid-rinsed and autoclaved polycarbonate bottles. Water samples were kept dark at in situ temperature for no more than 2 h before analysis.

Biotic and abiotic state variables. Duplicate samples were preserved with borax-buffered formaldehyde (2% final concentration) for microscopic counts of bacterial abundance. Cells were stained with 4,6-diamidino-2-phenylindole (DAPI) prior to analysis by epifluorescence microscopy (27). At least 200 cells or a minimum of 10 fields of view were analyzed.

Bacterial production was analyzed by [³H]leucine incorporation (36) using protein-to-biomass conversion factors according to the method of Simon and Azam (35).

Chlorophyll *a* was measured spectrophotometrically for cells retained on Whatman GF/F filters (nominal pore size, 0.7 μm). Frozen filters were extracted in 96% ethanol for 6 h, followed by removal of particulate matter by 0.2-μm-membrane filtration. Chlorophyll *a* absorbance was determined at 665 nm with corrections for suspended solids at 750 nm (15). Dissolved organic carbon was analyzed by high-temperature catalytic oxidation on a Shimadzu TOC analyzer as previously described (7).

Nucleic acid extraction. Bacterial cells for DNA extraction were captured by vacuum filtration (<30 kPa) of various volumes of lake water (150 to 500 ml) on 0.2-μm membrane filters (Supor, Gelman). Filters were immediately frozen and stored at -80°C until analysis. Bead beating and solid-phase extraction were used to isolate nucleic acids for PCR-based assays as detailed elsewhere (8). The quantity and average size of extracted nucleic acids were determined by 1% agarose gel electrophoresis (for more details, see reference 8). In short, gels were stained with ethidium bromide and extracted nucleic acids were quantified by comparison against a low-DNA-mass ladder (Invitrogen, Carlsbad, CA) using Gel-Pro Analyser version 3.1 (Media Cybernetics, Inc., Silver Spring MD). The concentration of genomic DNA in the extracts ranged from 2 to 20 ng μl⁻¹.

Q-PCR. Q-PCR reactions were prepared using Platinum SYBR Green qPCR SuperMix-UDS (Invitrogen, Carlsbad, CA) in 200-μl flat-lid PCR strips and an MX3000P thermocycler (Stratagene, La Jolla, CA). Results were analyzed with MxPro-Mx 3000P software (Version 3.00; Stratagene, La Jolla, CA). Reactions (20-μl volumes) contained 10 μl 2× Platinum SYBR Green PCR master mix, 250 nM of each primer (final concentration), 5 μl of template (2 to 20 ng DNA), 1 μl 20× bovine serum albumin, and UV-treated and 2-μl-filter-sterilized ultrapure Q-grade water. For each sample, two individual replicated Q-PCRs were performed using two different primer pairs. Primers used for amplification of class *Flavobacteria* 16S rRNA genes were 27f (5'-AGR GTT TGA TCM TGG CTC AG -3') (42) and 588 r (5'-GGA CCC TTT AAA CCC AAG -3') (modified from that described in reference 45). PCR was performed using a thermocycling program consisting of an initial step of 2 min at 95°C, followed by 45 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s with continuous acquisition of SYBR Green fluorescence signal. Total concentration of bacterial 16S rRNA genes was assessed using primers 27f (42) and 519r (5'-GWA TTA CCG CGG CKG CTG -3') (21) and the same PCR assay as described above with the exception that an annealing temperature of 50°C was used. For all samples, melting profiles were done to check if the length of the resulting PCR product was homogenous. Positive controls and titers for the Q-PCR assay included genomic DNA from a *Flavobacteria* isolate obtained from Lake Valentuna (isolate VA6, EF078979). This genomic DNA was diluted in 10-fold steps from 50 ng to 0.5 pg, and triplicates of each dilution were analyzed in parallel with the lake samples. Negative controls included samples lacking template DNA. The assay was not only tested with dilution series of the *Flavobacteria* strain, but also, a mixed environmental sample (Ekoln 13/08-2003) was serially diluted to three different concentrations. Simple regressions of the cycle threshold values for the DNA standards versus the log-transformed starting copy number were plotted for both the universal primer pair (total number of 16S rRNA genes) and the 16S rRNA genes targeted by the *Flavobacteria*-specific primer pair.

To provide information about reproducibility and sensitivity of the Q-PCR (37), the *r*² value, the amplification efficiency, and the slope were reported for each standard curve (Table 1). Finally, values were corrected for their dilution factor and averaged to calculate the relative amount of *Flavobacteria* compared to the total bacterial 16S rRNA gene copies in a given sample. Results from the clone libraries corroborate the specific amplification of the class *Flavobacteria* with primer 588r (see Results, below). Absolute numbers of *Flavobacteria* were determined by multiplying the bacterial cell abundance (DAPI counts) and the relative contribution of the flavobacterial gene copy number to the total 16S rRNA gene copy number. Regression analysis revealed a significant relationship between the cycle threshold values of the Q-PCR using general bacterial primers and the number of bacteria used for DNA extraction (*r*² = 0.35; *P* < 0.001).

TABLE 1. Regression coefficient (*r*²), amplification efficiency (*E*), and slope of four standard curves for real-time PCR amplification of rRNA genes^a

Sample or isolate analyzed	<i>r</i> ² value	<i>E</i> value	Slope
<i>Bacteria</i>			
VA 6	0.99	69.3	4.372
EK13/8	0.99	70.4	4.321
<i>Flavobacteria</i>			
VA 6	0.98	78.8	4.142
EK13/8	0.99	71.6	4.262

^a Either primer pair 27f and 519r (*Bacteria*) or pair 27f and 588r (*Flavobacteria*) was used. Standard curves for the environmental sample (EK13/8) and isolate VA 6 (EF078979) are shown.

T-RFLP. T-RFLP analysis of *Flavobacteria* 16S rRNA genes was used to assess the composition of the *Flavobacteria* communities. *Flavobacteria* 16S rRNA genes were amplified from mixed genomic samples using PCR with the bacterial primer 27f (42) and the class *Flavobacteria*-specific primer 588r (modified from reference 45).

The forward primer was labeled with hexachlorofluorescein at the 5' end (MWG Biotech) to enable fluorescence detection of terminal restriction fragments (ribotypes). Eight replicate PCRs of 20 μl (each) were amplified for each sample in a Stratagene Robocycler (Stratagene, La Jolla, CA) under the following PCR conditions: initial denaturation at 95°C for 8 min, followed by 20 cycles of amplification (1 min at 95°C, 1 min at 55°C, and 1 min at 72°C) and a final 4-min extension at 72°C.

Pseudoterminal fragments were eliminated by digestion of single-stranded DNA generated in the PCR with mung bean nuclease (6). The PCR products were then purified and concentrated using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). For each sample, aliquots containing approximately 50 ng of PCR product were digested with the endonuclease HhaI for at least 16 h according to instructions supplied by the manufacturer (Invitrogen). Fluorescently labeled fragments were separated by size in an ABI 3700 96-capillary sequencer running in GeneScan mode (Applied Biosystems, Foster City, CA). T-RFLP electropherograms were analyzed with the freeware GenScanView 4 (CRIBI group [http://grup.cribi.unipd.it]) using 1% of the total peak area as a lower cutoff for ribotypes to be included in the comparative analysis.

PCR and cloning. Two clone libraries from each lake were constructed from PCR products based on the bacterial primer 27f (42) and either bacterial primer 1492r (5'-GGYTACCTTGTTACGACTT-3') (21) or the *Flavobacteria*-specific primer 588r (modified from reference 45). The detailed procedures for generation of clone libraries using the universal bacterial primer pair are given elsewhere (8).

For PCR using *Flavobacteria*-specific primers, between 5 and 20 ng of genomic DNA from each sample was added to five replicate 20-μl reaction mixtures with PCR conditions as described for T-RFLP analysis. Replicate PCRs were pooled and precipitated with ethanol and sodium acetate (30), followed by agarose gel purification as described elsewhere (8). From each PCR product, 5 to 10 ng was cloned into the pCR 4-TOPO vector and transformed into competent *Escherichia coli* One Shot TOP10 using the TOPO TA cloning kit for sequencing as recommended by the manufacturer (Invitrogen, Carlsbad, CA).

All flavobacterial clone libraries (47 clones for each library) were screened for gene variants using a previously described high-resolution T-RFLP method (8). In contrast to the T-RFLP fingerprinting approach (described above), this method is based on parallel use of two endonucleases to cut a 16S rRNA gene fragment carrying different fluorescence tags at the two terminal ends. This procedure separates operational taxonomic units (OTUs) with 16S rRNA gene sequence similarities ranging from 73 to 99.9% for different taxonomic groups (8).

Sequencing and phylogenetic analysis. At least one clone was sequenced for each OTU. Additional clones, chosen randomly, were also sequenced for the more abundant OTUs. From the universal clone libraries, a total of 558 clones were sequenced (for more details, see reference 8). From the four *Flavobacteria* clone libraries a total of 89 clones were sequenced on an ABI 3700 96-capillary sequencer (Applied Biosystems) using the primer M13f and the BigDye Terminator kit, version 3.1 (Applied Biosystems). Four sequences were ambiguous and hence discarded. All other sequences were high-quality reads of ~550 bases. The RDPII Chimera Check program was used to identify sequences with a high

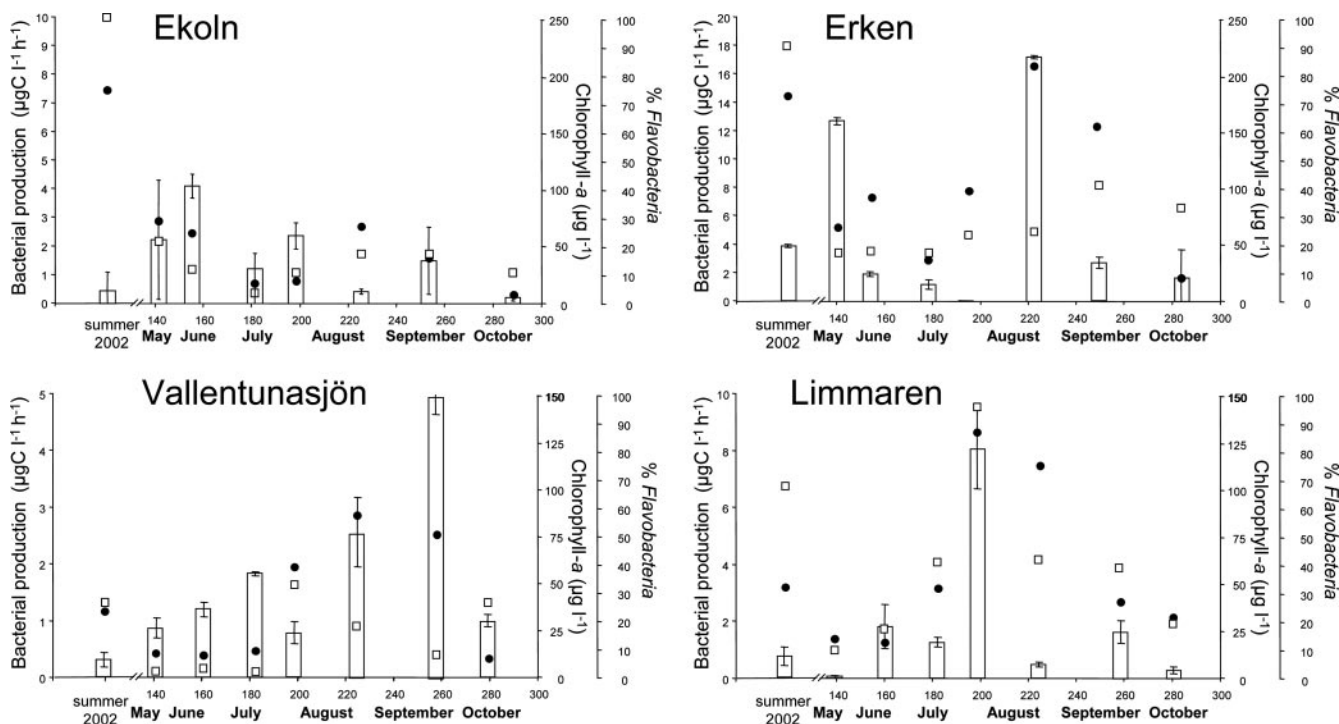


FIG. 1. Flavobacterial 16S rRNA genes as a percentage of total bacterial 16S rRNA genes (% *Flavobacteria*; bars), changes in bacterial production (filled circles), and chlorophyll *a* (open squares) in four lakes. All values are averages from triplicate analyses, and error bars indicate standard deviations.

probability of being chimeric. All cloned sequences were compared to GenBank entries using BLAST (Basic Local Alignment Tool; July 2006) in order to select reference sequences and obtain a preliminary phylogenetic affiliation of the clones. All sequences were imported into ARB and automatically aligned using the integrated aligner tool and the fast aligner option, followed by manual alignment of the sequences to closely related sequences with the secondary structure of rRNA taken into account. Phylogenetic analyses were performed with ARB (38). Trees were constructed using maximum likelihood, and tree topology was confirmed by maximum parsimony with 100 bootstrap replications and neighbor-joining with Jukes-Cantor distance correction (see reference 8 and references therein).

Data analyses. For T-RFLP, the relative abundance of ribotypes was estimated from the peak area in percentage of total peak area. Relative ribotype peak areas were binned according to fragment length (~1 bp). To investigate the degree of similarity between different *Flavobacteria* communities, T-RFLP patterns were converted to a binary (0/1) matrix. A dissimilarity matrix (D_{xy}) was calculated from the binary data with the equation $D_{xy} = 1 - 2N_{xy}/(N_x + N_y)$, where N_x and N_y represent the number of peaks present either in sample x or y , respectively, and N_{xy} is the number of peaks present in both samples (7). Cluster analysis using the unpaired-pair-group-method-using-average-linkages algorithm was carried out in Statistica (Statsoft, Tulsa, OK). The Mantel test was used to correlate the dissimilarity matrix of bacterial community composition with dissimilarity matrices of chlorophyll *a* and bacterial production. Instead of using the mean, replicated measurements were pooled prior to ordinary least-square regression and correlation analyses to minimize the effects of measurement errors. We estimated the strength of the linear relationship (r^2) and tested the association between dependent and independent variables (P value).

Nucleotide sequence accession numbers. All sequences were deposited in GenBank under accession numbers EF060976 to EF061064.

RESULTS

Limnology of the sampled lakes. The four lakes are similar in their chemical and physical features (8). Dissolved organic carbon concentrations varied little over season in each lake (coefficient of variance for Lake Ekoln, 10.4%; for Lake Erken,

1.8%; for Lake Vallentunasjön, 6.6%; for Lake Limmaren, 1.7%), and there were only minor differences between the four studied systems, with averages for the entire study ranging from 9.8 to 14.6 mg C liter⁻¹ (see Table S1 in the supplemental material). The chlorophyll *a* concentration was used as an approximation of phytoplankton biomass. The highest variation (50-fold) of the chlorophyll *a* concentration was observed for Lake Ekoln, and this lake also had the highest absolute chlorophyll *a* value (250 µg liter⁻¹) (Fig. 1). Microscopic inspection revealed that this bloom was almost exclusively (>80%) composed of *Microcystis aeruginosa* colonies. In Lake Erken and Lake Limmaren, chlorophyll *a* at peak bloom was five- to sevenfold higher than the average for all samples. For the other lakes, microscopic analysis also confirmed that phytoplankton biomass was mainly comprised of *Cyanobacteria* during the summer period, with a dominance of *Gloeotrichia echinulata* in Lake Erken and *Aphanizomenon* spp. in Lake Limmaren. Summer phytoplankton communities in Lake Vallentunasjön consisted of *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*, and *Microcystis* spp., with summer chlorophyll *a* levels three- to sevenfold higher than the chlorophyll *a* values measured in spring and autumn. Peaks of cyanobacterial blooms were accompanied by high abundances of heterotrophic bacteria, ranging from 10¹⁰ to 10¹¹ cells liter⁻¹ in the different lakes (see Table S1 in the supplemental material).

Diversity of class *Flavobacteria* in productive lakes. In the present study, the diversity of the class *Flavobacteria* was first analyzed by screening universal bacterial clone libraries for each of the four lakes. Samples for these libraries were obtained during cyanobacterial bloom events in the year 2002.

These four bacterial clone libraries were dominated by *Cyanobacteria*, although *Bacteroidetes* and *Proteobacteria* were also abundant (8). Of 1,461 clones, 105 were affiliated with the class *Flavobacteria*. The percentages of clones affiliated with *Flavobacteria* in bacterial clone libraries were 1.6% for Vallentunasjön, 2.7% for Lake Ekoln, 5.4% for Lake Erken and 23% for Lake Limmaren.

Clone libraries based on 16S rRNA genes amplified with primers specific to *Flavobacteria* were also prepared and analyzed for each lake. These libraries were prepared from samples collected in the summer of 2003 (Ekoln, 17/7; Erken, 13/8; Limmaren, 18/7; Vallentunasjön, 30/6). A total of 181 clones from the flavobacterial clone libraries were screened by high-resolution T-RFLP screening as described for the bacterial clone libraries (8). Twenty-four unique OTUs were detected, with 44 of the clones belonging to a single abundant OTU (B86; see Fig. S1 in the supplemental material). For B86, both T-RFLP fingerprinting and high-resolution T-RFLP screening revealed the same operationally defined group. In contrast, B88, operationally defined in the community T-RFLP fingerprint, represents a diverse group of *Flavobacteria* species as revealed by sequencing and high-resolution T-RFLP screening and includes several freshwater isolates (e.g., *Flavobacterium columnare*, *Flavobacterium succinicans*, and *Flavobacterium aquatile*). Using high-resolution T-RFLP screening, a total of five OTUs could be identified within B88. Similarly, clones that grouped into OTU B90 could be divided into two distinct groups (see Fig. S1 in the supplemental material).

Phylogenetic analyses and rarefaction analyses (Fig. 2) of the four clone libraries revealed a wide diversity of *Flavobacteria* in the four productive lakes. A few freshwater *Flavobacteria*, which have 16S rRNA sequences similar to those of the OTUs detected in the present study, have previously been isolated and characterized (2, 39). For example, *F. columnare* is a well-described fish pathogen, and *Flavobacterium limicola*, which has been isolated from freshwater sediments, can degrade various organic polymers.

Dynamics of class *Flavobacteria*. The proportion of the bacterial community affiliated with *Flavobacteria* varied substantially both among lakes and over time (Fig. 1). The highest relative abundance was observed during the senescence and decline of a cyanobacterial bloom in Lake Erken (with *Flavobacteria* contributing to nearly 100% of the bacterioplankton community). Additional periods where the bacterioplankton community was dominated by *Flavobacteria* were observed during summer in both Lake Limmaren (approximately 80%) and Lake Vallentunasjön (approximately 90%) (Fig. 1).

To increase the phylogenetic resolution of our PCR assay, T-RFLP fingerprinting was applied to follow the dynamics of operationally defined populations (ribotypes). T-RFLP results were compared by estimating the relative peak areas of individual ribotypes on different sampling occasions. Generally, two ribotypes, B88 and B99, were dominant in the T-RFLP electropherograms (Fig. 3). These populations were also mainly responsible for the observed *Flavobacteria* blooms, here defined as episodes where the contribution of *Flavobacteria* to total bacterioplankton exceeded 30%. During these blooms, multiple populations of *Flavobacteria* were often detected. In Lake Erken, ribotype B88 contributed more than 50% of the total 16S rRNA gene pool during the senescing phase of a

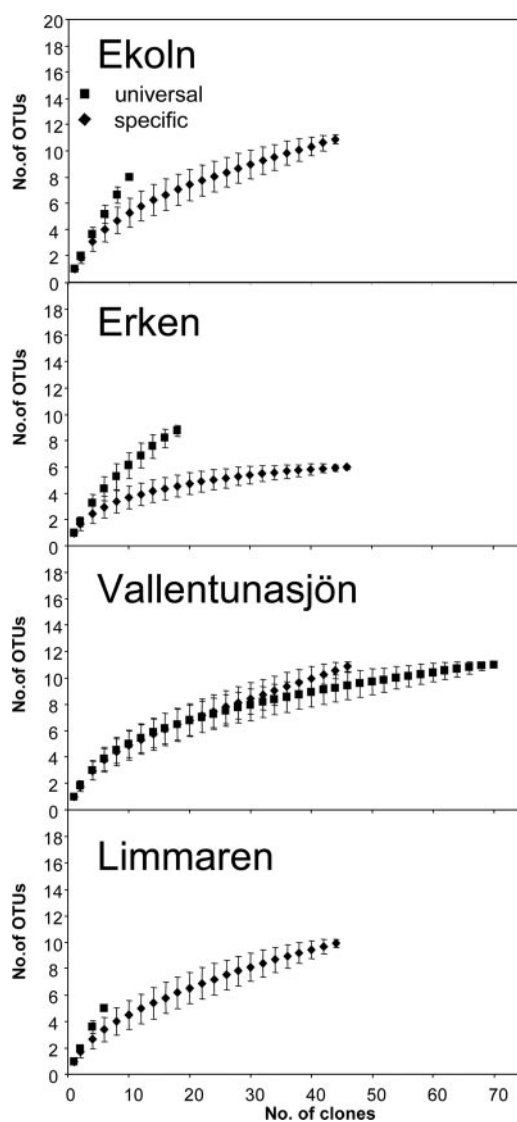


FIG. 2. Rarefaction analyses of the eight clone libraries based on high-resolution terminal restriction fragment length polymorphism screening. Two clone libraries using either universal primers (27f and 1492r) or flavobacterium-specific primers (27f and 588r) are plotted in each graph.

cyanobacterial bloom. In Lake Vallentunasjön, the combined contribution of B88 and B99 to the bacterial 16S rRNA gene pool exceeded 50% during a bloom of filamentous cyanobacteria at the beginning of August. Also, in Lake Limmaren samples from late July, the *Flavobacteria* community was dominated by these two ribotypes during a filamentous cyanobacterial bloom. Ribotype B88 was observed, usually in high relative abundances, in all samples except in spring samples of Lake Vallentunasjön. Also, ribotypes B90, B99, and B86 could be detected in more than 50% of the samples and at least twice in each lake. These operationally defined populations were also the only ones detected in the clone libraries constructed from the four lakes, and they represent diverse lineages, as indicated in the phylogenetic tree (see Fig. S1 in the supplemental material). In addition, 29 rare or occasionally occurring

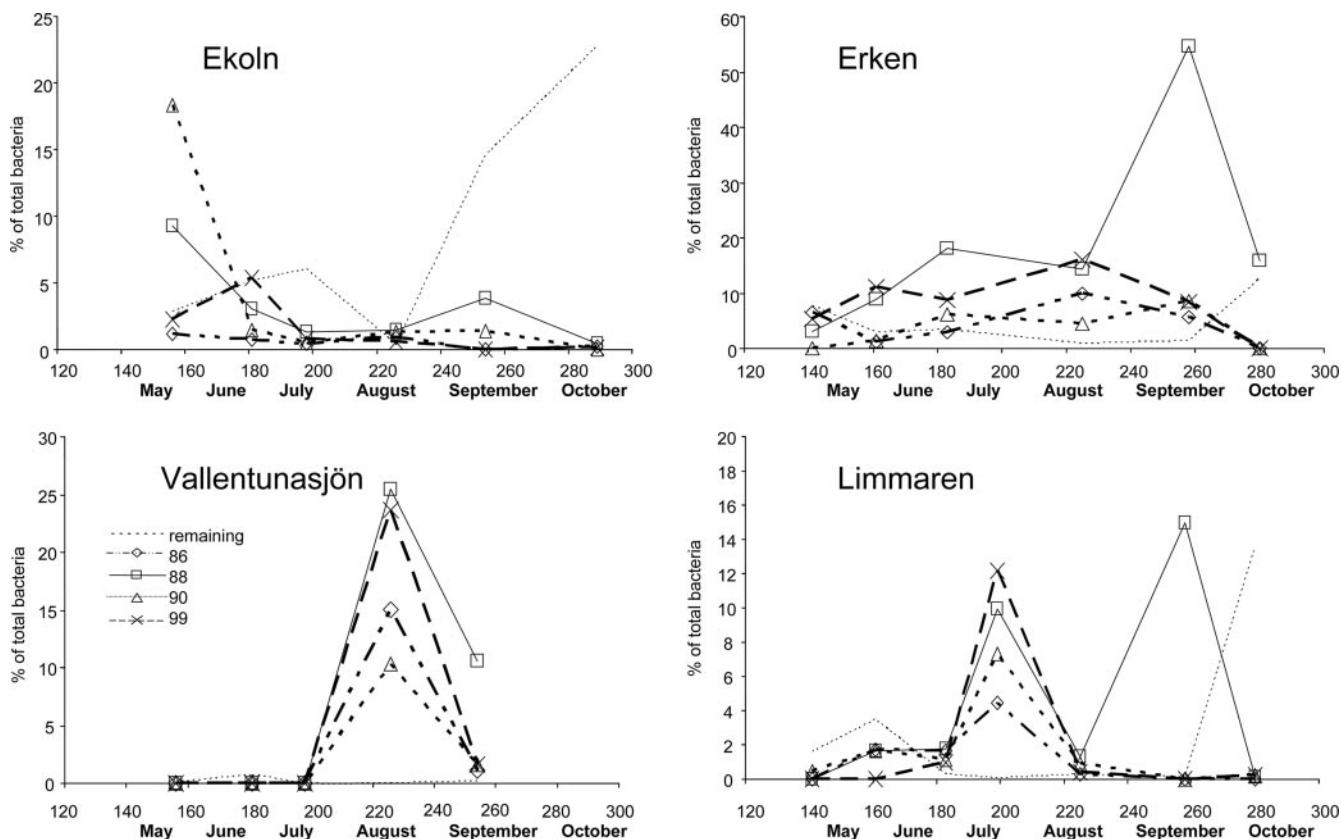


FIG. 3. Temporal dynamics of dominant operationally defined *Flavobacteria* populations (terminal restriction fragments B86, B88, B90, and B99) and sum of all other peaks (remaining) from T-RFLP profiles of HhaI-digested PCR-amplified DNA from four lakes. y axis represents relative peak intensity multiplied by relative contribution of *Flavobacteria* to the total bacterial community. Phylogeny of each terminal restriction fragment is shown in Fig. S1 in the supplemental material.

ribotypes were also detected in the 4 lakes. These populations were infrequently detected but contributed substantially to the relative peak areas in single autumn samples (Fig. 3).

DISCUSSION

In this study, we observed highly dynamic microbial communities, reflected by blooms of various *Flavobacteria* ribotypes in four eutrophic lakes. These blooms could be linked to bacterial production maxima and in some cases to cyanobacterial blooms (Fig. 1).

To determine the abundance of *Flavobacteria*, flavobacterium-specific primers and universal bacterial primers were applied in a Q-PCR. The flavobacterial primer 588r was modified to feature one mismatch at the 3' end (guanine instead of thymidine) for most known freshwater *Flavobacteria*, with the exception of one group (indicated by B0 in Fig. S1 in the supplemental material). 16S rRNA genes from members of group B0 have two mismatches to primer 588r and were not amplified in the *Flavobacteria* class-specific PCR, whereas other *Flavobacteria* targets containing only the 3' mismatch were amplified. At the same time, this modification prevents nonspecific amplification of the more than 9,000 bacterial non-targets in Ribosomal Database Project II, release 9, that feature only a single mismatch to the 5' end of the original probe 588r (see Table S2 in the supplemental material). This was

demonstrated by amplification of several single-mismatch *Flavobacteria* targets and lack of amplification of DNA from strains with two mismatches (data not shown). A regression between Q-PCR standard curves obtained using either the modified or unmodified primer 588r was highly significant, indicating that the two primers performed similarly in the quantification, except for the lower efficiency of the modified primer (see Table S2 and Fig. S2 in the supplemental material). The standard curves obtained from both isolate VA6 and an environmental sample suggested good linearity and no effect of dilution on quantification (Table 1). It is well known that 16S rRNA gene copy numbers can vary between different bacterial taxa and thus bias absolute quantification of bacterial abundance (see reference 37 and references therein). Also, amplification of plastid 16S rRNA genes can lead to an overestimation of total bacterial 16S rRNA genes. Results from the four bacterial clone libraries indicate that this bias is minor, since only between 1 and 5.5% of the clones in these libraries were affiliated with plastids (8). Comparison of the rarefaction curves obtained from the different libraries shows that the bacterial clone libraries exhibited steeper OTU rarefaction curves than the flavobacterial clone libraries for three of the lakes (Fig. 1). This corroborates that the *Flavobacteria* class-specific primers target a lower richness of OTUs within the *Flavobacteria* than the universal *Bacteria* primers (e.g., ribotype B0; see Fig. S1 in

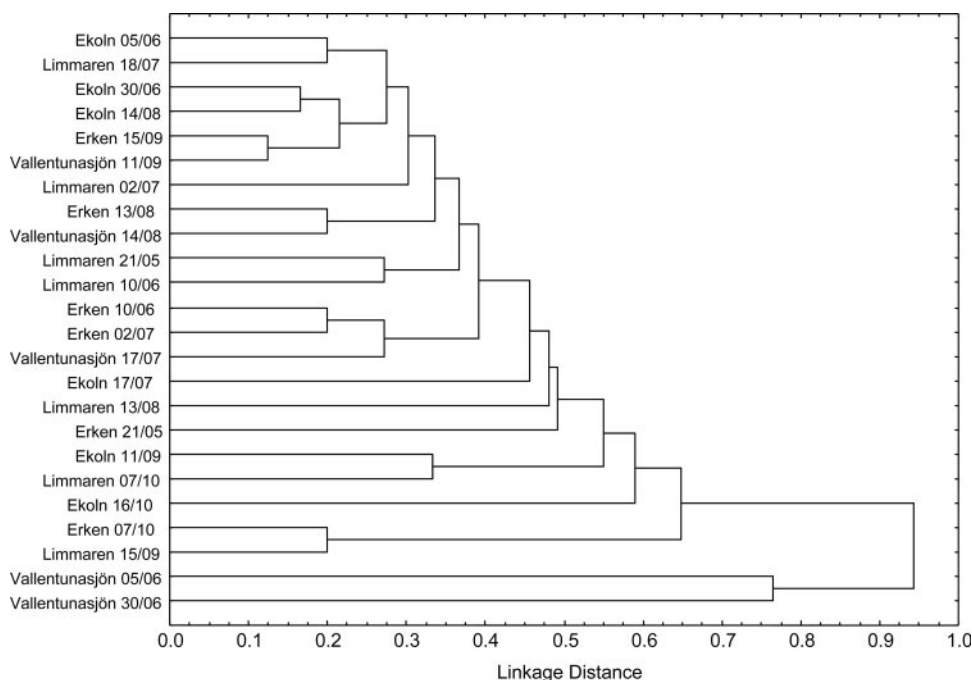


FIG. 4. Cluster analysis of flavobacterial communities. Unweighted pair-group tree diagram of 24 samples that were analyzed by T-RFLP fingerprinting and subsequent statistical analyses as described elsewhere (8).

the supplemental material). However, since the clone libraries were constructed from different samples, it is not possible to make any direct estimates of the fraction of the *Flavobacteria* community that escape detection. Still, as an example, none of the *Flavobacteria* clones detected in the bacterial clone libraries of Vallentunasjön would have been amplified with the flavobacterium-specific primer (two mismatches or more to the modified primer 588r).

With these limitations in mind, estimated 16S rRNA genes of *Flavobacteria* contributed on average 16% to the total 16S rRNA gene pool for Lake Ekoln, 35% for Lake Erken, 18% for Lake Limmaren, and 27% for Lake Vallentunasjön. Hence, our study reveals proportions of *Flavobacteria* in freshwater bacterioplankton communities similar to those observed in previous studies (10, 12, 19, 24, 33, 34, 47). In several samples from the four lakes, *Flavobacteria* contributed more than 30% to the total 16S rRNA gene pool. High proportions and abundances of single heterotrophic bacterial populations have been described previously as a result of high grazing pressure (25). Several other studies suggest that phytoplankton blooms and high grazing pressure can favor members of the *Bacteroidetes* division (for example, see references 24, 33, and 34).

Multivariate statistical analyses, like principal component analysis and partial least-squares projection to latent structures (data not shown), did not result in any satisfactory model which could explain the dynamics of the class *Flavobacteria*. An exception was that the contribution of *Flavobacteria* seemed to follow the trends of bacterial production determined from leucine incorporation (Fig. 3). Correlation and ordinary least-square regression analyses using bacterial production as the independent variable and estimates of absolute abundance of *Flavobacteria* as the dependent variable (replicate measurements

were pooled prior to analyses) showed a significant linear relationship ($r^2 = 0.42$; $P < 0.001$; $n = 32$). There was also a significant relationship between bacterial production and total bacterial abundance ($r^2 = 0.40$; $P < 0.001$; $n = 32$). This suggests that members of the class *Flavobacteria* are indeed favored by conditions that prevail during episodes of high heterotrophic activity and enhanced growth, but by no means does it imply that *Flavobacteria* are generally more competitive than other bacterioplankton under such conditions. The significant linear relationship between the chlorophyll *a* concentration and bacterial production ($r^2 = 0.50$; $P \ll 0.001$; $n = 32$) suggests that phytoplankton-derived organic matter is one of the main drivers of bacterial growth in the four studied lakes. A weak relationship between chlorophyll *a* and estimates of absolute abundance of *Flavobacteria* was observed ($r^2 = 0.18$; $P = 0.017$; $n = 32$). Previous studies also indicate that *Flavobacteria* can be very abundant during phytoplankton blooms in marine systems (1, 10) and in freshwater environments (47). Further evidence that *Flavobacteria* are abundant during phytoplankton blooms, particularly during cyanobacterial blooms in freshwater environments, comes from high frequencies of 16S rRNA genes affiliated with this class in PCR-based gene surveys of bloom-associated communities and consortia linked to cyanobacterial colonies (8, 9, 20, 28). However, it is difficult to make any general conclusions about the dynamics of the class *Flavobacteria* during phytoplankton blooms from either the present or previous studies (1, 26, 28, 41, 47), since results are inconclusive.

Bacteria not only are regulated by autochthonous resources (3), but they are also tightly linked to allochthonous resource inputs (7, 40) and controlled by both eukaryote and virus predation in the aquatic environment (4, 5, 11, 26, 31–34, 43).

Laboratory experiments suggest that certain groups of bacteria are more susceptible to flagellates grazing (16) and viral lysis (32) than others. Simek and colleagues (33, 34) reported that under enhanced grazing pressure, *Cytophaga-Flavobacteria* were overrepresented in grazing-resistant filamentous bacterial morphotypes (25).

Another explanation for the weak relationship between *Flavobacteria* and environmental variables may be provided by the physiological and ecological diversity within the class. Even if T-RFLP analysis was applied to reveal dynamics at the subclass level (Fig. 3), the observed ribotypes do not represent operational taxonomic units that are monophyletic (see Fig. S1 in the supplemental material) and it is not likely that they are ecologically and physiologically uniform. It should be emphasized that even organisms with identical 16S rRNA genes can exhibit different physiologies and inhabit different niches (14, 29). Nevertheless, cluster analysis of T-RFLP patterns using binary data for dissimilarity analysis (Fig. 4) suggested a highly dynamic *Flavobacteria* community with low similarities between lakes and over seasons. Samples from different lakes and at different time points clustered together, giving no support for distinct *Flavobacteria* community characteristics for a certain lake or a certain seasonal succession stage. The average similarity of all samples collected from the four lakes was 44%, and the average similarity of samples collected from the same lake was 51% for Lake Ekoln, 55% for Lake Erken, 42% for Lake Limnaren, and 24% for Lake Vallentunasjön. Hence, it could not be concluded whether the greatest component of variation was related to differences either among lakes or over time (see also Fig. 4). The *Flavobacteria* community of Lake Vallentunasjön was the most variable among the four lakes. This could be a result of morphology, since Lake Vallentunasjön is the shallowest of the four lakes (8), and its bacterial community may be more influenced by wind-driven sediment resuspension and a high content of suspended particles. Additional statistical analysis using a Mantel test revealed no correlation between chlorophyll *a* and bacterial community composition. A significant correlation between dissimilarity matrices of bacterial production and bacterial community composition ($r = 0.131$; $P = 0.02$) was observed, suggesting that not only *Flavobacteria* abundance but also the composition of the class *Flavobacteria* is related to bulk bacterial growth conditions. Since bacterial production is equal to the amount of organic matter that can be transformed into bacterial biomass, the covariation further indicates that resource availability is likely to regulate *Flavobacteria* community composition. This is consistent with other studies that have shown correlations between resources and community composition (5, 26).

To conclude, eutrophic lakes harbor highly dynamic bacterioplankton communities which feature temporary blooms of *Flavobacteria* populations. The correlation between *Flavobacteria* dynamics and bacterial production suggests that the availability of resources is likely to shape heterotrophic bacterial seasonal succession.

ACKNOWLEDGMENTS

We thank Edith Schallmeiner for assistance with setting up the Q-PCR assay.

This work was funded by the Swedish Research Council for Environment, Agricultural Sciences and Special Planning and the Swedish Research Council (grants to S.B.).

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