Seasonal patterns of the bacterioplankton community composition in a large mesotrophic lake

Walter Zwisler, Natascha Selje, Meinhard Simon*

Institute for Chemistry and Biology of the Marine Environment, University of Oldenburg, 26111 Oldenburg, Germany

ABSTRACT: The composition of the bacterioplankton community in mesotrophic Lake Constance was studied from April 1996 to December 1997 by fluorescence *in situ* hybridization (FISH) with group specific oligonucleotide probes and denaturing gradient gel electrophoresis (DGGE). Between 10 and 59 % of the DAPI-stained cells were detected as *Bacteria* with means of 38 ± 12 , 27 ± 10 and 23 ± 6 % at 3, 20, and 50 m depth, respectively. In the euphotic zone at 3 m depth, β -*Proteobacteria* always constituted highest proportions of the bacterial community detected by FISH and constituted 6 to 23 % of the DAPI-stained cells with a mean of 12 %. Proportions of bacteria belonging to the *Cytophaga/Flavobacteria* (CF) cluster and the α -*Proteobacteria* at this depth were 5.4 ± 2.5 and 4.1 ± 3.5 %, respectively. Proportions of β -*Proteobacteria* decreased at 20 and 50 m, whereas that of α -*Proteobacteria* remained constant and that of the CF-cluster increased to 7.4 %, such that they comprised similar proportions as β -*Proteobacteria* at 50 m depth. γ -*Proteobacteria* were always of minor importance and constituted only 2.2 ± 1.3 % of the DAPI cell counts. Cumulative proportions of all detected bacterial groups constituted 75 % of the *Bacteria* counts at 3 m, 84 % at 20 m and 99 % at 50 m. The DGGE analysis of the bacterioplankton community showed seasonal and vertical variations of the banding patterns even though several bands occurred throughout the study period and at all depths. A cluster analysis revealed distinct bacterial clusters of the phytoplankton spring bloom and the later period and of epilimnetic and hypolimnetic waters. The sequence analysis of the excised bands indicated that all groups which were detected by FISH were represented in the patterns. Sequence similarities of the retrieved sequences to known isolates were <90 to 96.1 %, and to other phylotypes as close as 99 %. Phylotypes of β -*Proteobacteria* and *Actinobacteria* of the typical banding patterns affiliated closely with recently described clusters of typical freshwater bacteria. In addition, we identified phylotypes of the *Holophaga/Acidobacterium* and *Nitrospira* phylum during the stratified period in samples from 50 m and during mixing from all 3 depths.

KEY WORDS: Bacteria · DGGE · Fluorescence *in situ* hybridization · Lake Constance · *Actinobacteria* · *Nitrospira* · *Holophaga*

— Resale or republication not permitted without written consent of the publisher —

INTRODUCTION

During the last 2 decades bacterioplankton has been identified and characterized as an integral component of pelagic ecosystems. In many studies heterotrophic bacteria have been shown to process large proportions of the organic carbon fixed by phytoplankton primary production. Depending on the trophic state and season, this proportion varies from 20 to >60 % (Cole et al. 1988, Simon et al. 1998a). An inherent presumption of all studies which focused on the cycling of organic matter

by bacterioplankton is that the latter are considered as an entity or black box without any further differentiation with respect to its taxonomic composition. In order to obtain a better understanding of the growth dynamics, spatio-temporal variations and controlling factors of bacterioplankton communities, it is of great importance to elucidate their taxonomic composition as well.

During the last decade powerful molecular biological methods, based on various aspects of the analysis of nucleic acids, became available to examine the taxonomic composition of natural bacterial communities.

*Corresponding author. Email: m.simon@icbm.de

The 2 approaches most widely used are rRNA-targeted fluorescence *in situ* hybridization (FISH; Amann et al. 1995) and denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA gene fragments (DGGE) (Muyzer et al. 1998). The first approach allows a quantitative analysis of the composition of natural prokaryotic communities. Using probes of various specificity, 40 to 80% of the bacterioplankton cells in freshwater and marine environments have been detected (Glöckner et al. 1999, Šimek et al. 1999, 2001, Simon et al. 1999, Brümmer et al. 2000, Eilers et al. 2000). In freshwater systems, usually β -*Proteobacteria* dominate, and cells of the *Cytophaga/Flavobacteria* cluster also comprise high proportions, whereas in marine environments members of the *Cytophaga/Flavobacteria* cluster, followed by α - and γ -*Proteobacteria*, constitute largest proportions of the detectable bacterial cells. β -*Proteobacteria* appear to be absent in marine environments except in estuarine and coastal areas. Despite the availability of the FISH approach for more than 8 yr, surprisingly few studies on the spatio-temporal dynamics of bacterioplankton communities are available. In order to better understand their growth dynamics and controlling factors, more such studies are needed.

The DGGE approach is attractive because it allows a rapid qualitative analysis of the phylogenetic structure of bacterial communities. Its application revealed rather stable seasonal as well as pronounced temporal and spatial variations of bacterial communities in aquatic systems (Lindström 1998, 2000, Konopka et al. 1999, Lebaron et al. 1999, Hollibaugh et al. 2000, Riemann et al. 2000, Riemann & Winding 2001). So far, spatio-temporal dynamics of pelagic bacterioplankton communities in large lakes have not been investigated by DGGE. Given the limitations of the DGGE and FISH approaches, we assume that we will obtain a much better insight into the spatio-temporal dynamics and controls of the composition of the bacterioplankton community if we apply both approaches simultaneously.

The aim of our study was to investigate the spatio-temporal dynamics of the bacterioplankton community in Lake Constance, a large mesotrophic lake, over a 2 yr period. β -*Proteobacteria* always constituted the largest proportion of the bacterial community as identified by FISH. The DGGE and cluster analysis further revealed that distinct bacterial clusters occurred seasonally and vertically. The sequence analysis of the 16S rRNA gene fragments revealed that in the epilimnion few β -*Proteobacteria*, and also *Actinobacteria*, with high GC content existed. Additionally, few *Nitrospira*- and *Holophaga/Acidobacterium*-related bacteria with high GC contents were detected in the hypolimnion.

MATERIALS AND METHODS

Study site and sampling. Lake Constance is a warm-monomictic mesotrophic lake adjacent to Germany, Switzerland and Austria with a total area of 472 km². Maximum and mean depths are 253 and 101 m, respectively. The plankton ecology of the lake has been studied extensively during the last decade (Gaedke 1998, Häse et al. 1998, Simon et al. 1998a). Samples were collected between April 1996 and December 1997 in weekly to monthly intervals at the center of Lake Überlingen, a fjord-like arm in the north-western area of the lake. The samples were transferred into opaque plastic jars and further processed in the lab within 2 hr. Temperature and chl *a*, determined spectrophotometrically after hot ethanol extraction (Häse et al. 1998), were available from the data base of the special collaborative program 'Cycling of Matter in Lake Constance' (SFB-248).

Total cell abundance. Bacterial abundance was determined by epifluorescence microscopy (Porter & Feig 1980). Duplicate subsamples of 1 to 2 ml were stained with DAPI for 15 min in the dark, filtered through 0.2 μ m black Nuclepore filters and enumerated under a Nikon epifluorescence microscope (Labophot 2). The coefficient of variation (CV; standard deviation/mean) of the 20 fields of view counted was always <0.11.

FISH. We performed FISH with rRNA-targeted oligonucleotide probes of triplicate subsamples as described in Glöckner et al. (1996). Briefly, 3 to 5 ml samples were filtered onto black 0.2 μ m Nuclepore filters, fixed for 30 min with a freshly prepared 4% paraformaldehyde solution, rinsed twice with 3 ml of phosphate buffer saline (PBS), pH 7.2, and kept at 4°C in the dark until hybridization within 3 wk. For hybridization, filters were cut into thirds and placed onto glass slides. The following oligonucleotide probes were applied to duplicates: EUB338 specific for the domain *Bacteria* (Amann et al. 1990), ALF1b, BET42a, GAM42a (α -, β - and γ -*Proteobacteria*; Manz et al. 1992) and CF319a (bacteria of the *Cytophaga/Flavobacteria* cluster; Manz et al. 1996). Hybridization with the oligonucleotide probes was done at 46°C for 90 min in the dark, followed by washing with PBS for 15 min at the same temperature. Thereafter, the samples were stained by DAPI and the bacteria enumerated by epifluorescence microscopy. The CV from counts of 10 fields of view on triplicate filter pieces was always <0.15.

Nucleic acid extraction and PCR amplification of 16S rRNA gene fragments. For genomic DNA extraction, bacterial biomass of 100 to 200 l from 3 m depth was concentrated by tangential flow filtration with a 0.1 μ m hollow fiber cartridge (Amicon ProFlux M 12). In samples from the depth profiles, bacterial biomass of 150 ml samples was collected on 0.2 μ m Nuclepore

filters. The biomass concentrates were stored at -30°C for up to 6 mo until DNA extraction. DNA was extracted by a slightly modified standard hot phenol protocol. Therefore, the biomass was resuspended in 500 μl TENP-buffer (50 mM TRIS, 20 mM EDTA, 100 mM NaCl, 2% PVPP, pH 8) (Picard et al. 1992) and 100 μl of a 20% sodium dodecyl sulphate (SDS) solution. Protein was removed by a hot phenol and phenol/chloroform extraction (chloroform = chloroform/isoamylalcohol: 24/1). After DNA precipitation with isopropanol, the nucleic acid was redissolved in 50 μl Tris-ethylenediaminetetraacetic acid (TE)-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4°C .

PCR was performed in 50 μl assays with a GENIUS thermal cycler (Techne). 16S rRNA gene fragments were amplified using the primer set 341F (5'-GC-clamp-CCT ACG GGA GGC AGC AG-3'; GC-clamp: 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') and 907Re (5'-CCG TCA ATT C[A/C]T TTG AGT TT-3'; Wawer et al. 1997, Muyzer et al. 1998). All PCRs were conducted with 1 to 2 μl of DNA template solution (10 to 30 ng DNA) in buffer supplied by the *Taq* polymerase manufacturer (Pharmacia). The final volume of 50 μl contained 200 μM of the dNTP-mixture (Pharmacia), 0.5 μM of each primer and 2 units of *Taq* polymerase in double distilled H_2O . After an initial denaturing step (2 min, 94°C) a 'touchdown' PCR (Don et al. 1991) was performed in which the annealing temperature was set to 65°C (50 s) and decreased 0.5°C every cycle until 55°C was reached (20 cycles) and 12 more cycles were carried out. The polymerization step was performed at 72°C for 2 min. PCR products were examined by standard agarose gel electrophoresis (1% agarose, $1\times$ TAE buffer [40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA]). DNA was visualized by ethidium bromide fluorescence.

DGGE. PCR products were analyzed by DGGE with a DCode™ System (BioRad) as described elsewhere (Muyzer et al. 1996, Teske et al. 1996). DGGE was performed in a 6% (wt/vol) polyacrylamide gel with a denaturing gradient from 20 to 50% denaturants (100% means 7 M urea and 40% [vol/vol] formamide). Twenty μl of the PCR products (600 ng DNA) were loaded on each lane of the gel. On each gel the PCR product of a bacterial isolate from Lake Constance (Ub41) was used as a standard. After electrophoresis for 5 h at constant 200 V and a temperature of 60°C , gels were stained either with ethidium bromide (0.5 mg l^{-1} ; 20 min) or SYBR® Gold (0.1 $\mu\text{l ml}^{-1}$; 40 min; Molecular Probes) and photographed on a UV transilluminator with a black and white Polaroid Camera. Several series of parallel gels stained by both dyes yielded consistently similar banding patterns of equal intensity.

A cluster analysis of the DGGE banding patterns was performed using the software GelCompare II, Version

2.5 (Applied Maths, St. Martens-Latem, Belgium). We applied 5 to 20% background subtraction depending on the signal-to-noise ratio of the corresponding gel. Patterns were compared curve-based using Pearson correlation as similarity coefficient and UPGMA (unpaired group method of analysis) to generate the dendrogram. We used the position tolerance optimization option of the software to fit the curves to the best possible matching. From the given cophenetic correlation values and the similarity values for the internal standards, the most likely result was chosen.

For sequence analyses pieces of selected DGGE bands were cut out of the gel and stored for 48 h at 4°C in 50 μl of ultrapure water (K-persulfate-oxidized Milli-Q water). After centrifugation (1 min, $8000\times g$), 1 μl of the supernatant was used for another PCR amplification as described above. Because this second-step PCR product was used for cloning or direct sequencing, the 341F primer had no GC-clamp on its 5'-end.

Cloning and sequencing. For the sequence analysis, DNA of a single phylotype is necessary. To ensure this the PCR products of the excised bands were cloned with the PCR-Script™ Amp Cloning Kit (Stratagene) into Epicurian Coli® XL1-Blue MRF'Kann supercompetent cells. The amplified plasmid was purified with the ClearCut™ Miniprep Kit (Stratagene). All steps in these procedures followed the instruction manuals of the manufacturer.

Sequence reactions were performed by the professional sequencing company GATC Biotech AG. Sequence analysis was done with the software package ARB (Strunk et al. 1998). The phylogenetic analysis was performed with the ARB software package (available at www.arb-home.de; Strunk et al. 1998). An unrooted tree was constructed using parsimony, neighbor-joining, and maximum-likelihood analysis. The backbone tree calculated using maximum-likelihood analysis and almost full length 16S rRNA gene sequences (>1300 bp). A total of 148 sequences from different families, as suggested in the prokaryotic representative listing in the Ribo-somal Database Project II (RDP 2000, available at <http://rdp.cme.msu.edu/html/>), and sequences of closest relatives as found by BLAST search, were used for the backbone tree. A filter was constructed for every phylogenetic group in focus of the analysis. Hence, alignment positions, at which less than 50% of sequences of the corresponding data set had the same residues, were excluded from the calculations to prevent uncertain alignments within highly variable positions. Shorter sequences and sequences of the DGGE bands were added afterwards by maximum parsimony using the same filter. Sequences are available at GenBank under the accession numbers AF337192-AF337214 (see Table 2).

Fig. 1. Temperature at 5 m depth and chl *a* concentrations at 3 m depth in Lake Constance between January and December 1996

RESULTS

In 1996, the temperature started to increase in April and reached more than 15°C in July such that the lake became thermally stratified. Highest temperatures of around 20°C occurred in August. Thereafter, the lake continuously cooled down and reached 6°C in December (Fig. 1). In 1997, the onset of the warming started 3 wk later but proceeded rather similarly to the previous year. The thermal stratification persisted longer in late summer and fall such that in December the lake was still warmer than 8°C in the upper 30 m.

The phytoplankton spring bloom in 1996 developed between mid-April and mid-May and reached chl *a* concentrations of 22.4 µg l⁻¹ (Fig. 1). It was terminated by heavy grazing of daphnids and followed by the clear water-phase in June. In July and August, 2 further phytoplankton blooms were recorded. In the following year, the spring bloom developed 3 wk later and its peak reached only 50% of the chl *a* concentration recorded in 1996 (data not shown). In summer, chl *a* concentrations remained lower than in the previous year and did not exceed 6 µg chl l⁻¹. In October and late November, however, 2 further phytoplankton blooms occurred.

Bacteria and FISH

In 1996, total bacterial numbers varied between 1.6 and 6.1 × 10⁶ ml⁻¹ at 3 m (Fig. 2A). Highest numbers were reached at the first chlorophyll peak of the spring bloom in the second half of April. Further peaks occurred at the end of the spring bloom during the clear-water phase and in late August. In 1997, the seasonal patterns of the bacterial numbers at 3 m, ranging between 1.4 and 6.0 × 10⁶ ml⁻¹,

were basically similar to those in 1996 (data not shown).

Cells of the domain *Bacteria* detected by probe EUB338 ranged from 0.5 to 2.6 × 10⁶ ml⁻¹, and accounted for 40.3% of the DAPI counts in 1996, and 34.6% in 1997, at 3 m as an annual mean (Table 1). Both percentages were not significantly different from each other (*U*-test, *p* < 0.01). Highest fractions constituted 59.3 and 55.4% of the DAPI cell counts in both years, respectively (Table 1). There was no systematic covariation of the fraction of *Bacteria* detected by EUB338 with bacterial numbers, chlorophyll concentrations or temperature. *β*-*Proteobacteria* constituted highest numbers and proportions of all bacteria detected at 3 m and comprised 11 to 13% of the DAPI cell counts in both years, equivalent to 35% of the *Bacteria* counts (Fig. 2D, Table 1). Maxima and minima during the spring bloom, the clear-water phase, and in summer often covaried with those of the DAPI cell counts. The linear regression analysis between the DAPI cell counts and EUB cell counts in 1996 and 1997 yielded an *r*² of 0.51 (*n* = 22, *p* < 0.05) and of 0.98 (*n* =

Fig. 2. Bacterioplankton numbers at 3 m depth in Lake Constance between April and November 1996. (A) Numbers of DAPI-stainable cells and *Bacteria* (EUB), (B) *Cytophaga/Flavobacteria* (CF), (C) *γ-Proteobacteria* (GAM), (D) *β-Proteobacteria* (BET), (E) *α-Proteobacteria* (ALF)

Table 1. Annual means $\pm$ SD and range of *Bacteria*, α -, β - and γ -*Proteobacteria* and *Cytophaga/Flavobacteria* as percent of DAPI-stainable cells and % *Bacteria* for 1996 and 1997 at 3 m depth in Lake Constance

	1996				1997			
	Mean % DAPI	Mean % <i>Bacteria</i>	Range % DAPI	Range % <i>Bacteria</i>	Mean % DAPI	Mean % <i>Bacteria</i>	Range % DAPI	Range % <i>Bacteria</i>
<i>Bacteria</i>	40.3 $\pm$ 11.4	–	15.5–59.3	–	34.6 $\pm$ 12.9	–	10.1–55.4	–
α - <i>Proteobacteria</i>	4.3 $\pm$ 4.6	11.6 $\pm$ 12.7	3.6–16.6	0.8–44.7	3.9 $\pm$ 2.5	12.5 $\pm$ 6.9	3.6–16.6	0.8–44.7
β - <i>Proteobacteria</i>	12.8 $\pm$ 4.7	33.8 $\pm$ 12.8	6.3–22.5	14.6–60.8	11.2 $\pm$ 3.8	34.9 $\pm$ 7.6	3.3–16.2	23.1–46.0
γ - <i>Proteobacteria</i>	2.6 $\pm$ 1.9	6.7 $\pm$ 5.6	0.4–7.5	1.0–23.8	1.8 $\pm$ 0.7	6.2 $\pm$ 3.1	0.9–3.4	2.8–12.9
<i>Cytophaga/Flavobacteria</i>	5.7 $\pm$ 3.3	14.2 $\pm$ 7.2	0.7–13.1	3.4–30.8	5.1 $\pm$ 1.8	18.9 $\pm$ 11.3	3.4–8.4	7.1–42.4

10, $p < 0.05$), respectively. On some occasions, such as during the early spring bloom and in early July, β -*Proteobacteria* accounted for as much as 55 to 60 % of the EUB counts. Cells of the *Cytophaga/Flavobacteria* cluster constituted between 4.3 and 8.7 % of the DAPI cell counts at 3 m except during the clear-water phase when they reached only 1 to 3 % (Fig. 2B). Between the end of October 1996 and March 1997 and in December 1997 they reached between 19 and 42 % of the *Bacteria* counts and constituted proportions nearly as high as those of β -*Proteobacteria*. As an annual mean, *Cytophaga/Flavobacteria* constituted 5.7 and 5.1 % of the DAPI cell counts in 1996 and 1997, respectively, which is equivalent to 14.2 and 18.9 % of the *Bacteria* counts (Table 1). α -*Proteobacteria* only reached substantial proportions of the bacterioplankton community after the clear-water phase from July until September. Except for a short time during this period, they constituted 7 to 16 % of the DAPI cell counts, equivalent to 20 to 40 % of the *Bacteria* counts, with a mean of 6.7 % (Fig. 2E). They were the second most abundant bacterial phylogenetic group during this period. As an annual mean α -*Proteobacteria* comprised around 4 % of the DAPI cell counts and 12 % of the *Bacteria* counts (Table 1). γ -*Proteobacteria* comprised always lowest proportions of all bacteria detected without any systematic patterns.

In May, August, October, November and December 1997 we also analyzed the community structure of the bacterioplankton by FISH at 20 and 50 m. DAPI cell counts systematically decreased with depth and reached around 1×10^6 cells ml^{-1} at 50 m. Accordingly, absolute numbers of bacteria detected by the *Bacteria* probe and group specific probes decreased with depth as well. Proportions of DAPI cell counts detected by the EUB-probe at 20 and 50 m were only 27 and 23 %, respectively (Fig. 3). Relative proportions of β - and γ -*Proteobacteria* did not change vertically. The proportions of α -*Proteobacteria*, however, significantly increased at 20 and 50 m as compared to 3 m and reached 22 % of the *Bacteria* counts. Cells of the *Cytophaga/Flavobacteria* cluster constituted signifi-

cantly higher proportions of *Bacteria* counts at 50 m as compared to 3 and 20 m and reached 32 %, the same proportion as β -*Proteobacteria*. The cumulative fraction of bacteria detected by the group-specific probes increased with depth and reached 99 % of the EUB counts at 50 m (Fig. 3).

Fig. 3. Proportions of *Bacteria* (% DAPI cell counts) and of α - (ALF), β - (BET) and γ -*Proteobacteria* (GAM) and of *Cytophaga/Flavobacteria* (CF) at 3, 20 and 50 m depth in Lake Constance (% *Bacteria*). Mean values $\pm$ SE from samples taken on 21 May, 26 August, 7 October, 4 November and 17 December 1997 are given. Percentage of *Bacteria* not detected by any of the probes applied is also given (not identified)

Fig. 4. Inverted DGGE gel stained with SYBR®-Gold of PCR-amplified 16S rRNA gene fragments from samples collected at 3 m depth in Lake Constance between 17 April and 10 September 1996. Numbers denote bands which were excised for sequence analysis and phylogenetic affiliation. Numbers of the bands refer to the numbering of the LCo clones. Ub41: reference strain

DGGE banding patterns and sequence analysis

The DGGE banding patterns of the 3 m samples showed seasonal variations but the majority of the bands occurred throughout the study period, such as Band 72 (Fig. 4). Ten to 15 bands were detected on each lane. The cluster analysis identified 2 major clusters, 1 of the phytoplankton spring bloom (17 April to 21 May), and 1 of the following period including the clear-water phase in June and summer until September. The cluster of the latter period is further subdivided into 1 subcluster including the clear-water phase in June and the period of late August until September at rather low chl *a* concentrations, and 1 including the period from late July until mid-August when the second chl *a* summer peak occurred.

A DGGE analysis was also conducted with samples from depth profiles between August and December 1997 (Fig. 5). The banding patterns of the 3 and 20 m samples of any given date were rather similar. In the 50 m samples, however, another prominent band occurred (Bands 18 and 23, Fig. 5). This band was also detected at 3 and 20 m depth in December. The cluster analysis showed that the 3 and 20 m sample of any given date clustered closely together and that 2 major clusters existed. One included all

samples from 3 and 20 m depth and that of 50 m of October. The 3 and 20 m samples of December and the 50 m sample of October formed a separate subcluster. The second cluster included the other 50 m samples.

The 16S rRNA gene fragments of the most prominent bands of the 3 m samples from various samples were cloned and sequenced (Clones LCo36 to 39, LCo70 to 76, LCo78 to 79, Fig. 4). Numbers of the bands are identical to the numbers of the LCo clones. The sequence analysis revealed that at least 9 different phylotypes were represented by these bands (Table 2, Fig. 6). Surprisingly, only 2 sequences originated from phylotypes of *β-Proteobacteria*, *Acidovorax* sp. and *Polynucleobacter necessarius* being the closest related isolates with sequence similarities of 96.0 and 96.7%. These phylotypes were even closer related to others from various freshwater lakes with sequence similarities

of 96.7 to 99.3% of the betaII cluster of Glöckner et al. (2000). One phylotype of *α-Proteobacteria* was retrieved which was related most closely to *Sphingomonas* sp. The 5 phylotypes associated to the *Cytophaga/Flavobacteria* cluster were related to *Cyclobacterium marinus* and *Flectobacillus* sp., but with sequence similarities of <93%. These phylotypes showed rather close sequence similarities with others, in particular from a lake in the Adirondack Mountains, USA (Hiorns et al. 1997) and within the cIII-cluster of Glöckner et al. (2000). From the bands migrating

Fig. 5. Inverted DGGE gel stained with ethidium bromide of PCR-amplified 16S rRNA gene fragments from samples collected at 3, 20 and 50 m depth in Lake Constance on 26 August, 16 September, 29 October and 17 December 1997. Numbers denote bands which were excised for sequence analysis and phylogenetic affiliation. Numbers of the bands refer to the numbering of the LCo clones. Ub41: reference strain

Fig. 6. Rooted phylogenetic tree of the sequenced phylotypes retrieved from the PCR-amplified 16S rRNA gene fragments of the DGGE analysis from Lake Constance and their next related isolates and phylotypes. A backbone tree based on maximum likelihood analysis was constructed with almost complete (>1300 nucleotides) sequences. Alignment positions at which less than 50 % of the corresponding sequences had the same base pairs were excluded. Sequences shorter than 1300 nucleotides were added with maximum parsimony using the same filter. The scale bar indicates 10 % estimated sequence divergence

Table 2. Clones from Lake Constance samples retrieved by DGGE, their phylogenetic affiliation and accession numbers, nearest known related isolates and phylotypes with sequence similarities and accession numbers according to BLAST search. Numbers of the bands in Figs. 4 & 5 refer to the numbering of the LCo clones. CF: *Cytophaga/Flavobacteria*. FuKu: Lake Fuchskuhle; GKS: Lake Gösseck; LD: Lake Lördrecht; ACK: Adirondack Mountains; ESR: Lake Esrom

Clone	Date	Depth (m)	Clone length (bp)	Category	Accession no.	Nearest known isolate (accession no.)	Similarity (%)	Nearest known phylotype (accession no.)	Similarity (%)
LCo75	30 Apr 1996	3	492	α -Proteobacteria	AF337211	RUB_R2A19 (AF121902) <i>Sphingomonas</i> sp. FO813 (AF235997)	96.1 96.1	FuKuN22 (AJ289994)	94.3
LCo74	30 Apr 1996	3	486	β -Proteobacteria	AF337210	UFZ-B530 (AF235013) <i>Acidovorax</i> sp.	96.0	GKS2-122 (AJ290026)	96.7
LCo76	30 Apr 1996	3	500	β -Proteobacteria	AF337212	<i>Polynucleobacter necessarius</i> (X93019)	96.7	LD17 (BSPZ99998) FuKuN33 (AJ289997) ACK L5 (U85122)	99.3 >97.1
LCo38	6 Aug 1996	3	515	CF	AF337204	<i>Flectobacillus</i> sp. (AJ011917)	91.7	ACK M36 (U85113)	98.3
LCo71	17 Apr 1996	3	459	CF	AF337207	<i>Flectobacillus major</i> (M62787)	85.1	ACK M36 (U85113)	97.6
LCo70	17 Apr 1996	3	513	CF	AF337206	<i>Cyclobacterium marinum</i> (M62788)	90.2	KEppib23 (AF188174)	95.3
LCo36	6 Aug 1996	3	461	CF	AF337202	<i>Cyclobacterium marinum</i> (M62788)	92.5	KEppib23, (AF188174)	94.0
LCo37	6 Aug 1996	3	486	CF	AF337203	<i>Cyclobacterium marinum</i> (M62788)	88.8	KEppib45 (AF188175)	93.5
LCo19	26 Aug 1997	50	186	<i>Holophaga/</i> <i>Acidobacterium</i>	AF337195	<i>Acidobacterium capsulatum</i> (D26171)	81.9	Uncultured soil bacteria SO13 (AF128677)	96.0
LCo18	26 Aug 1997	50	285	<i>Nitrospira</i>	AF337194	<i>Nitrospira moscoviensis</i> (AF155153)	92.3	Uncultured Green Bay ferromagnetic micronod (AF293012)	98.9
LCo23	29 Oct 1997	50	575	<i>Nitrospira</i>	AF337199	<i>Nitrospira moscoviensis</i> (AF155153)	95.0	Uncultured Green Bay ferromagnetic micronod (AF293012)	98.3
LCo79	30 Apr 1996	3	486	<i>Actinobacteria</i>	AF337214	' <i>Microthrix parvicella</i> ' (X89774)	86.5	Clone 1204-39 (AF289929)	96.1
LCo25	29 Oct 1997	50	558	<i>Actinobacteria</i>	AF337200	' <i>Microthrix parvicella</i> ' (X89774)	87.1	Clone 1404-31 (AJ22835) ESR8 (AF268292)	98.5 97.3

Table 2 (continued)

Clone	Date	Depth (m)	Clone length (bp)	Category	Accession no.	nearest known isolate (accession no.)	Similarity (%)	Nearest known phylotype (accession no.)	Similarity (%)
LCo20	26 Aug 1997	50	187	<i>Actinobacteria</i>	AF337196	' <i>Microthrix parvicella</i> ' (X89774)	87.2	Uncultured bacteria ARFS-33 (AJ277699)	94.3
LCo73	17 Apr 1996	3	471	<i>Actinobacteria</i>	AF337209	' <i>Microthrix parvicella</i> ' (X89774)	83.8	Uncultured bacteria ARFS-33 (AJ277699) Uncultured bacteria 404-29 (AJ289958)	94.3 94.2
LCo26	29 Oct 1997	50	564	<i>Actinobacteria</i>	AF337201	' <i>Microthrix parvicella</i> ' (X89774)	87.0	Uncultured bacteria 404-11 (AJ289955) Uncultured bacteria ARFS-33 (AJ277699)	94.8 94.4
LCo78	30 Apr 1996	3	485	<i>Actinobacteria</i>	AF337213	' <i>Nostocoida limicola II</i> ' (X85212)	93.5	ESR12 (AJ268296)	98.0
LCo72	17 Apr 1996	3	467	<i>Actinobacteria</i>	AF337208	' <i>Nostocoida limicola II</i> ' (Y14597)	93.8	Uncultured bacteria (AJ231070) ESR12 (AJ268296)	98.1 97.5
LCo17	26 Aug 1997	20	290	<i>Actinobacteria</i>	AF337193	' <i>Nostocoida limicola II</i> ' (Y14597)	92.4	Uncultured bacteria (AJ231070) FuKuN105 (AJ289968) ESR12 (AJ268296)	95.2 94.8 94.5
LCo16	26 Aug 1997	3	267	<i>Actinobacteria</i>	AF337192	<i>Geodermatophilus</i> sp. (X92364)	92.1	FuKuN105 (AJ289968) Uncultured bacteria (AJ231070)	95.1 95.1
LCo39	6 Aug 1996	3	522	<i>Actinobacteria</i>	AF337205	<i>Actinobacterium</i> sp. (AJ229252)	90.6	Uncultured bacteria 1405-41 (AJ289938)	97.3
LCo22	29 Oct 1997	20	511	<i>Actinobacteria</i>	AF337198	<i>Cellulomonas</i> sp. (AB023361)	90.6	Uncultured bacteria 1405-9 (AJ289938) Uncultured bacteria 25-5-69 (AJ289953)	99.5 98.2
LCo21	29 Oct 1997	3	515	<i>Actinobacteria</i>	AF337197	<i>Cellulomonas</i> sp. (AB023361)	90.9	Uncultured bacteria 25-5-69 (AJ289953) Uncultured bacteria clone 404 (AJ007646)	98.5 97.4

farthest in the gels, 5 phylotypes of the high GC *Actinobacteria* were retrieved which clustered with *Cellulomonas* sp./'*Nostocoida limicola II*' and '*Microthrix parvicella*' (Table 2, Fig. 6) and within the cluster hgcl of Glöckner et al. (2000). Phylotypes LCo71, LCo75 and LCo38 migrated in the gel in a similar way, but belonged to 2 completely different phylogenetic lineages, LCo75 to α -*Proteobacteria* and the others to *Cytophaga/Flavobacteria* (Fig. 6, Table 2). The same was true for phylotype LCo74, belonging to β -*Proteobacteria* and phylotypes LCo70 and LCo37, which were identified as *Cytophaga/Flavobacteria*. These results show that the structure of the bacterioplankton community was not as stable as inferred from the DGGE banding patterns and included bacteria not detected by the group-specific probes applied.

We also sequenced 16S rRNA gene fragments of 10 bands of the depth profiles (bands and clones LCo16 to 23, LCo25 and 26, Table 2, Fig. 6). The bands occurring only at 50 m between August and October were identified as representing phylotypes of the *Nitrospira* group (LCo18 and 23) and the *Holophaga/Acidobacterium* group (LCo19). The other bands represented phylotypes of *Actinobacteria* from the 2 groups mentioned above.

DISCUSSION

In surface waters at 3 m depth, roughly 40% of the DAPI-stainable cells were detected as *Bacteria* by probe EUB338, but at 20 and 50 m depth only 27 and 23% were *Bacteria*. At 3 and 20 m at least 75% of the *Bacteria* were identified as members of α -, β - and γ -*Proteobacteria* and the *Cytophaga/Flavobacteria* cluster, and at 50 m even >95%, indicating that a substantial fraction of the bacterioplankton community could be characterized with the group-specific phylogenetic probes applied. Detecting cells by FISH depends on the amount of ribosomes per cell, the accessibility of the target molecule, and the fluorescence signal (Amann et al. 1995). The amount of ribosomes per cell is a positive function of the bacterial growth rate (Kemp et al. 1993), and thus an important constraint of detecting microbes by FISH in oligotrophic natural environments (Alfreider et al. 1996). The accessibility of the target molecule depends largely on the fixation of the cells and is usually done by a 4% paraformaldehyde solution for Gram-negative bacteria. The fluorescence signal has been continuously improved by applying more intensive fluorescence dyes (Southwick et al. 1990, Kenzaka et al. 1998) and polyribonucleotide probes (DeLong et al. 1999) or helper probes (Fuchs et al. 2000, Glöckner et al. 2000). The application of polyribonucleotide and helper probes signifi-

cantly improved the probe signal such that the great majority of the DAPI-stainable planktonic bacteria could be detected. In fact, applying probes and helper probes specific for cluster hgcl of the *Actinobacteria*, Glöckner et al. (2000) demonstrated that bacteria of this cluster are important members of the bacterioplankton in freshwater lakes constituting 25 to 50% of the DAPI-stainable cells. Bacteria of this cluster were not yet detected by more traditional probes and neglected so far in FISH studies. Taking them into account rises the total fraction of DAPI-stainable bacteria in lakes to >95% (Glöckner et al. 2000). We used the paraformaldehyde fixation procedure and CY3 as one of the most intense fluorochromes, and thus procedures widely applied. Therefore, and because we did not apply the helper probe-mix for the hgcl cluster of the *Actinobacteria*, it is not surprising that our detection rate by the EUB probe is at the lower end of the range found in other studies carried out in lacustrine environments applying similar types of probes (Pernthaler et al. 1998, Glöckner et al. 1999, Jürgens et al. 1999, Šimek et al. 1999, 2001). It is comparable to the detection rate of a study in the North Sea (Eilers et al. 2000) but lower than that of a study in the Southern Ocean (Simon et al. 1999). It is also lower than detection rates of bacteria associated with natural organic aggregates, activated sludge flocs or in river biofilms (Wagner et al. 1993, Grossart & Simon 1998, Manz et al. 1999, Brümmer et al. 2000, Schweitzer et al. 2001, Simon et al. 2002). Bacteria in these habitats are bigger than free-living cells and contain more ribosomes. The lower fraction of cells detected in our study at 20 and 50 m are presumably due to the lower growth rates and thus lower numbers of ribosomes as compared to 3 m depth (Simon et al. 1998b).

Proportions of the various bacterial groups exhibited relatively low fluctuations even though pronounced spatio-temporal fluctuations in absolute cell numbers occurred. An important question concerns possible controlling factors explaining the spatio-temporal dynamics of the bacterioplankton community. These factors include substrate availability, hydrolysis and uptake, growth properties and temperature adaptation, phage infection and grazing. We made extensive statistical analyses of the temporal dynamics of the bacterioplankton groups detected by FISH with that of temperature, chl *a*, phytoplankton biomass, bacterial biomass production, and concentration and uptake of monosaccharides, which were measured simultaneously (Simon et al. 1998a, Bunte & Simon 1999). None of these parameters showed significant correlations to dynamics of either of the bacterial groups, emphasizing the fact the group specific probes target rather broad and physiologically non-homogenous bacterial groups.

β -*Proteobacteria* were the most abundant bacterial group identified by FISH in Lake Constance during the growing season, and were closely correlated to the DAPI cell counts. This notion is consistent with comparable studies in other freshwater systems including lakes, microscopic and macroscopic limnetic aggregates, and river biofilms (Grossart & Simon 1998, Pernthaler et al. 1998, Glöckner et al. 1999, Šimek et al. 1999, 2001, Brümmer et al. 2000, Brachvogel et al. 2001, Schweitzer et al. 2001). Despite their great significance, limited information is available on the relative distribution of specific populations of β -*Proteobacteria* in lacustrine environments. In a high mountain lake, 2 probes targeting narrow clusters related to *Rhodferax fermentans* and *Alcaligenes* sp. detected between <10 and >50% of all β -*Proteobacteria*, with an annual mean of 25% (Pernthaler et al. 1998). In line with these findings, Šimek et al. (2001) found that β -*Proteobacteria* of a narrow cluster, also related to *Rhodferax fermentans* and targeted with a single probe, constituted the single most abundant bacterial group in a grazer-free mesocosm. In a small eutrophic lake, a narrow cluster of β -*Proteobacteria* including *Comamonas acidovorans*, *C. testosteroni*, *Acidovorax temperans*, and *A. facilis* comprised proportions of 5 to 70% of the bacterioplankton community annually (Höfle et al. 1999). Bacterial communities on lake snow aggregates are also largely dominated by β -*Proteobacteria* and consist of relatively few members as well. Schweitzer et al. (2001), applying 3 probes of narrow clusters related to *Duganella zoogloeoides* (formerly *Zoogloea ramigera*), *Hydrogenophaga* sp. and *A. facilis*, detected between 40 and 60% of all β -*Proteobacteria* on lake snow aggregates in Lake Constance. The abundance of these species in the free-living bacterioplankton community, however, was below the detection limit (W. Zwisler & M. Simon unpubl. data). It is interesting, though still puzzling, that β -*Proteobacteria* of a rather limited phylogenetic diversity are such a dominating group of the heterotrophic freshwater bacterioplankton. Obviously, they share common genetic properties which are advantageous for thriving in such ecosystems. So far, no such traits specific to β -*Proteobacteria* are known which would explain their high abundance.

Cytophaga/Flavobacteria were the second most abundant bacterioplankton group identified by FISH in Lake Constance, with highest abundances under conditions when polymeric substrates and recalcitrant polymeric substrates were dominant such as during phytoplankton blooms and in deeper water layers and during winter. Labile organic substrates such as amino acids, proteins and carbohydrates are primarily produced in the course of phytoplankton blooms during the growing season in euphotic water layers, and

exhibit much lower concentrations in aphotic water layers and during winter (Simon 1998, Bunte & Simon 1999, Rosenstock & Simon 2001). *Cytophaga/Flavobacteria* are known to degrade polymeric substrates of labile and rather recalcitrant nature and to comprise many meso- and psychrophilic species (Reichenbach 1992). Hence, these notions are consistent with the spatio-temporal distribution of *Cytophaga/Flavobacteria* found in the present study. *Cytophaga/Flavobacteria* have also been identified as important members of bacterial communities in other aquatic environments including lakes, rivers, and marine systems (Pernthaler et al. 1998, Šimek et al. 1999, Simon et al. 1999, Brümmer et al. 2000, Cottrell & Kirchman 2000). In river biofilms, they constituted highest proportions during winter (Brümmer et al. 2000). Šimek et al. (1999, 2001) reported that under enhanced protozoan grazing pressure, *Cytophaga/Flavobacteria* dominated the grazing-resistant bacterial filaments.

α -*Proteobacteria* constituted substantial proportions between July and September and occasionally were the second most important bacterial group during this period. Even though the proportion of this group relatively increased at 20 and 50 m depth as compared to 3 m, its proportion always remained lower than that of β -*Proteobacteria* and *Cytophaga/Flavobacteria*. As the probe Alf1b also matches some *Nitrospira* sequences, we cannot rule out the possibility that this probe also detected *Nitrospira*-related cells at 20 and 50 m where we detected sequences of this phylotype. There is very limited information available on the occurrence of this subclass of *Proteobacteria*, making it difficult to explain their growth patterns. In a mesocosm study examining grazing effects of protozoans and daphnids on the structure of limnetic bacterioplankton, Jürgens et al. (1999) reported that α -*Proteobacteria* constituted enhanced proportions when daphnids were absent and protozoan grazing was high. Similar observations, but not as clear-cut, were made by Šimek et al. (1999) examining size-fractionated grazing on bacterioplankton in a eutrophic reservoir. Schweitzer et al. (2001) and Knoll et al. (2001) experimentally examined the temporal succession of α - and β -*Proteobacteria* on lake snow aggregates and precursor microaggregates in Lake Constance, and found within the initial days high, but thereafter decreasing, proportions of α -*Proteobacteria*. On natural microaggregates in this lake, α -*Proteobacteria* were absent, supporting the idea that microaggregates represent particulate organic matter depleted greatly of labile organic matter (Brachvogel et al. 2001). Hence, there is some evidence that limnetic α -*Proteobacteria* comprise bacteria which prefer rather labile organic matter, although more studies need be carried out to shed more light on the specific properties of members of this subclass of *Proteobacteria*.

The DGGE and sequence analyses showed that the major phylogenetic groups detected by FISH consisted of a limited number of bacteria and phylotypes. In addition, phylotypes of 3 other phylogenetic groups not targeted by FISH were detected. The cluster analysis indicated that distinct bacterial communities existed in the epi- and hypolimnion and during the major seasonal phases such as the spring bloom and the summer period. These phases were also characterized by enhanced cell numbers of *β-Proteobacteria* and *Cytophaga/Flavobacteria*. Hence, the DGGE banding patterns, the cluster analysis and the sequencing results complement the FISH data with the group specific probes, also by revealing dominant phylotypes of the various groups detected by FISH.

Mesocosm studies with nutrient additions and different grazing impacts experimentally demonstrated that changes in substrate supply and grazing can lead to substantial changes in the bacterial community composition (Lebaron et al. 1999, van Hannen et al. 1999, Riemann et al. 2000, Riemann & Winding 2001). Substrate supply and grazing impact exhibit pronounced changes seasonally in Lake Constance (Simon et al. 1998a, Bunte & Simon 1999, Rosenstock & Simon 2001), which obviously lead to changes in the bacterial community composition not detected by FISH but by the cluster analysis of the DGGE banding patterns. In contrast to our study, stable seasonal DGGE patterns were found in a Swedish lake (Lindström 1998). In another study in various lakes (Lindström 2000), positive correlations between grazing by microzooplankton and chrysophytes and the composition of the bacterial community were found, suggesting that grazing was a significant factor controlling the bacterioplankton community composition.

The sequence analysis of the phylotypes excised from the DGGE bands revealed rather different relationships to identified isolates and phylotypes. Only few phylotypes of the *α*- and *β-Proteobacteria* were retrieved, whereas a much higher diversity of the *Cytophaga/Flavobacteria* and *Actinobacteria* was found. Sequence similarities of the phylotypes to known isolates ranged from <90 to 96.7 %, with lowest similarities in the *Cytophaga/Flavobacteria*, *Actinobacteria* and *Holophaga/Acidobacterium* cluster, and highest similarities within the *α*- and *β-Proteobacteria* and the *Nitrospira* phylum. Sequence similarities between our phylotypes of the *α*- and *β-Proteobacteria* were rather similar for known isolates and phylotypes. The phylotypes we retrieved from the *Cytophaga/Flavobacteria*, *Holophaga/Acidobacterium* and *Actinobacteria* cluster, however, were much closer related to phylotypes from other freshwater lakes than to known isolates, thus complementing other recent studies (Glöckner et al. 2000, Zwart et al. 2002). Phylotype LCo76 affiliated

with the betaII cluster of Glöckner et al. (2000), unique to uncultured freshwater *β-Proteobacteria*. The next relatives of phylotype LCo74 of *β-Proteobacteria*, even though still fairly distantly related, were found to be important components of the bacterioplankton in a eutrophic lake (Höfle et al. 1999). Thirteen of our phylotypes were identified as *Actinobacteria*. Five of them appeared within typical bands present throughout the study period at 3 m depth, and thus were integral components of the bacterioplankton community in Lake Constance. The phylotypes LCo16, 17, 21, 22, 39, 72, and 78 of the high GC *Actinobacteria* fall into the hgcl cluster of Glöckner et al. (2000). This cluster is unique to uncultured freshwater bacteria, including phylotypes from various European and North American mountain lakes, and Lake Baikal (Hiorns et al. 1997, Zwart et al. 1998, 2002, Glöckner et al. 2000, Riemann & Winding 2001), and thus presumably is a cluster of unique freshwater bacteria occurring worldwide. Interestingly, 5 of these phylotypes clustered particularly closely with those from Lake Baikal (Glöckner et al. 2000). The most closely related bacteria are *Cellulomonas* sp., *Geodermatophilus* sp., and '*Nostocoida limicola*', of which the latter occurs in activated sludge with only a few known strains (Liu et al. 2000). The other 7 phylotypes of the high GC *Actinobacteria* are related to the deep-branching cluster of '*Microthrix parvicella*'. This bacterium, even though only distantly related to the phylotypes we retrieved from Lake Constance, and of which only a few strains are known, also occurs in activated sludge (Blakall et al. 1995, 1996). Phylotypes of this cluster, related closely to our phylotypes, have also been detected in other freshwater lakes (Glöckner et al. 2000, Riemann & Winding 2001). We did not detect *Actinobacteria* by FISH and thus are unable to estimate their quantitative significance. According to the results of Glöckner et al. (2000) using FISH, these bacteria appear to be of great importance in freshwater lakes but could only be detected by applying helper probes in FISH analyses. An experimental study showed that *Actinobacteria* are grazing resistant (Pernthaler et al. 2001). Therefore, and in agreement with our DGGE results, we assume that they are a prominent component of the bacterioplankton in Lake Constance and obviously in other lakes as well.

Our sequence analysis further identified phylotypes which affiliate with the novel *Holophaga/Acidobacterium* and the *Nitrospira* phylum, from which only 3 and 2 isolates are known, respectively (Watson et al. 1986, Ehrlich et al. 1995, Spring et al. 2000). Bacteria of the *Holophaga/Acidobacterium* group have recently been found in sediments of Lake Baikal and Lake Constance (Spring et al. 2000). Obviously, bacteria of this novel phylum are also present in the hypolimnion. The *Nitrospira* phylum has been found to comprise com-

mon nitrite-oxidizers in waste-water treatment plants (Schramm et al. 1998, Okabe et al. 1999, Daims et al. 2000). *Nitrospira* phylotypes closely related to our phylotypes were recently retrieved from sediments of Green Bay, Lake Michigan, USA (Stein et al. 2001). As shown by the DGGE patterns, our phylotypes occurred at 50 m depth between August and October, and also in December at 3 and 20 m. This finding suggests that nitrite-oxidation in Lake Constance during the stratified period in the hypolimnion and during winter mixing throughout the water column is mediated predominantly by *Nitrospira*-like bacteria, and further that nitrite-oxidation in lacustrine environments in general, not only in waste-water treatment plants, is mediated largely by bacteria of this phylum.

In summary, the FISH results show that β -*Proteobacteria* constituted the highest proportions of the identified bacterioplankton community followed by *Cytophaga/Flavobacteria*. The DGGE banding patterns and cluster analysis of the bacterioplankton community composition in Lake Constance revealed differences between the phytoplankton spring bloom and later in the season. The sequence analysis showed that in addition to α - and β -*Proteobacteria* and the *Cytophaga/Flavobacteria* members of the high GC *Actinobacteria*, the *Nitrospira*-phylum and members of the *Holophaga/Acidobacterium* group also occur regularly in the lake. Thus, the DGGE and sequence analysis complemented the FISH results by giving more detailed insights into the structure of the various bacterial groups detected by FISH, and by identifying phylotypes not targeted by the probes applied.

Acknowledgements. We very much appreciate constructive criticism by 3 anonymous reviewers on an earlier version of this manuscript. This work was supported by grant Si360/4-3 and the special collaborative program 'Cycling of Matter in Lake Constance' (SFB-248) from the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

- Alfreider A, Pernthaler J, Amann R, Sattler B, Glöckner FO, Wille A, Psenner R (1996) Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. *Appl Environ Microbiol* 62:2138–2144
- Amann R, Krumholz L, Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol* 172:762–770
- Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 59:143–169
- Blackall LL, Seviour EM, Cunningham MA, Seviour RJ, Hugenholtz P (1995) *Microthrix parvicella* is a novel, deep branching member of the actinomycetes subphylum. *Syst Appl Microbiol* 17:513–518
- Blackall LL, Stratton H, Bradford D, delDot T, Sjørup C, Seviour EM, Seviour RJ (1996) '*Candidatus Microthrix parvicella*', a filamentous bacterium from activated sludge sewage treatment plants. *Int J Syst Bacteriol* 46:344–346
- Brachvogel T, Schweitzer B, Simon M (2001) Dynamics and bacterial colonization of microaggregates in a large mesotrophic lake. *Aquat Microb Ecol* 26:23–35
- Brümmer IHM, Fehr W, Wagner-Döbler I (2000) Biofilm community structure in polluted rivers: abundance of dominant phylogenetic groups over a complete annual cycle. *Appl Environ Microbiol* 66:3078–3082
- Bunte C, Simon M (1999) Bacterioplankton turnover of dissolved free monosaccharides in a mesotrophic lake. *Limnol Oceanogr* 44:1862–1870
- Cole JJ, Findlay S, Pace ML (1988) Bacterial production in fresh and saltwater ecosystems: a cross system overview. *Mar Ecol Prog Ser* 43:1–10
- Cottrell M, Kirchman DL (2000) Natural assemblages of marine *Proteobacteria* and members of the *Cytophaga/Flavobacteria* cluster consuming low- and high-molecular weight dissolved organic matter. *Appl Environ Microbiol* 66:1692–1697
- Daims H, Nielsen PH, Nielsen JL, Juretschko S, Wagner M (2000) Novel *Nitrospira*-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: diversity and in situ physiology. *FEMS Microbiol Ecol* 33: 147–155
- DeLong EF, Trent Taylor L, Marsh TL, Preston CM (1999) Visualization and enumeration of marine planktonic *Archaea* and *Bacteria* by using polyribonucleotide probes and fluorescence in situ hybridization. *Appl Environ Microbiol* 65:5554–5563
- Don RH, Cox PT, Wainwright B, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res* 19:4008
- Ehrlich S, Behrens D, Lebedeva E, Ludwig W, Bock E (1995) A new obligately chemolithoautotrophic nitrite-oxidizing bacterium, *Nitrospira-moscoviensis* sp. nov. and its phylogenetic relationship. *Arch Microbiol* 164:16–23
- Eilers H, Pernthaler J, Glöckner FO, Amann R (2000) Culturability and in situ abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* 66:3044–3051
- Fuchs BM, Glöckner FO, Wulf J, Amann R (2000) Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. *Appl Environ Microbiol* 66:3603–3607
- Gaedke U (1998) The response of the pelagic food web to re-oligotrophication of a large and deep lake (L. Constance): evidence for scale-dependent hierarchical patterns? *Arch Hydrobiol Spec Issues Adv Limnol* 53:317–333
- Glöckner FO, Amann R, Alfreider A, Pernthaler J, Psenner R, Trebesius K, Schleifer KH (1996) An in situ hybridization protocol for detection and identification of planktonic bacteria. *System Appl Microbiol* 19:403–406
- Glöckner FO, Fuchs BM, Amann R (1999) Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. *Appl Environ Microbiol* 65:3721–3726
- Glöckner FO, Zaichikov E, Belkova N, Denisova L, Pernthaler J, Pernthaler A, Amann R (2000) Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of Actinobacteria. *Appl Environ Microbiol* 66: 5053–5056
- Grossart HP, Simon M (1998) Bacterial colonization and microbial decomposition of limnetic organic aggregates

- (lake snow). *Aquat Microb Ecol* 15:127–140
- Häse C, Gaedke U, Seifried A, Beese B, Tilzer MM (1998) Phytoplankton response to re-oligotrophication in large and deep Lake Constance: photosynthetic rates and chlorophyll concentrations. *Arch Hydrobiol Spec Issues Adv Limnol* 53:159–178
- Hiorns WD, Methé BA, Nierzwicki-Bauer SA, Zehr JP (1997) Bacterial diversity in Adirondack Mountain lakes as revealed by 16S rRNA gene sequences. *Appl Environ Microbiol* 63:2957–2960
- Höfle MG, Haas H, Dominik K (1999) Seasonal dynamics of bacterioplankton community structure in a eutrophic lake as determined by 5S rRNA analysis. *Appl Environ Microbiol* 65:3164–3171
- Hollibaugh JT, Wong PS, Murrell MC (2000) Similarity of particle-associated and free-living bacterial communities in northern San Francisco Bay, California. *Aquat Microb Ecol* 21:103–114
- Jürgens K, Pernthaler J, Schalla S, Amann R (1999) Morphological and compositional changes in a planktonic bacterial community in response to enhanced protozoan grazing. *Appl Environ Microbiol* 65:1241–1250
- Kemp PF, Lee S, LaRoch J (1993) Estimating the growth rate of slowly growing marine bacteria from RNA content. *Appl Environ Microbiol* 59:2594–2601
- Kenzaka T, Yamaguchi N, Tani K, Nasu M (1998) rRNA-targeted fluorescent in situ hybridization of bacterial community structure in river water. *Microbiology* 144:2085–2093
- Knoll S, Zwisler W, Simon M (2001) Bacterial colonization of early stages of limnetic diatom microaggregates. *Aquat Microb Ecol* 25:141–150
- Konopka A, Bercot T, Nakatsu C (1999) Bacterioplankton community diversity in a series of thermally stratified lakes. *Microb Ecol* 38:126–135
- Lebaron P, Servais P, Trousselier M, Courties C and 6 others (1999) Changes in bacterial community structure in sea-water mesocosms differing in their nutrient status. *Aquat Microb Ecol* 19:255–267
- Lindström ES (1998) Bacterioplankton community composition in a boreal forest lake. *FEMS Microbiol Ecol* 27:163–174
- Lindström ES (2000) Bacterioplankton community composition in five lakes differing in trophic status and humic content. *Microb Ecol* 40:104–113
- Liu JR, Burrell P, Seviour EM, Soddell JA, Blackall LL, Seviour RJ (2000) The filamentous bacterial morphotype '*Nostocoida limicola*' I contains at least two previously described genera in the low G+C gram positive bacteria. *Syst Appl Microbiol* 23:528–534
- Manz W, Amann R, Ludwig W, Wagner M, Schleifer KH (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. *Syst Appl Microbiol* 15:593–600
- Manz W, Amann R, Ludwig W, Vancanneyt M, Schleifer KH (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga-Flavobacter-Bacteroides in the natural environment. *Microbiology* 142:1097–1106
- Manz W, Wendt-Potthoff K, Neu TR, Szewzyk U, Lawrence JR (1999) Phylogenetic composition, spatial structure and dynamics of lotic bacterial biofilms investigated by fluorescent in situ hybridization and confocal laser scanning microscopy. *Microb Ecol* 37:225–237
- Muyzer G, Hottenträger S, Teske A, Wawer C (1996) Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA—a new molecular approach to analyse the genetic diversity of mixed microbial communities. In: Akkermans ADL, van Elsas JD, de Bruijn FJ (eds) *Molecular microbial ecology manual*, 2nd edn. Kluwer Academic Publishers, Dordrecht, p 1–23
- Muyzer G, Brinkhoff T, Nübel U, Santegoeds C, Schäfer H, Wawer C (1998) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: Akkermans ADL, van Elsas JD, de Bruijn FJ (eds) *Molecular microbial ecology manual*, 3rd edn. Kluwer Academic Publishers, Dordrecht, p 1–27
- Okabe S, Satoh H, Watanabe Y (1999) In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. *Appl Environ Microbiol* 65:3182–3191
- Pernthaler J, Glöckner FO, Unterholzner S, Alfreider A, Psenner R, Amann R (1998) Seasonal community and population dynamics of pelagic *Bacteria* and *Archaea* in a high mountain lake. *Appl Environ Microbiol* 64:4299–4306
- Pernthaler J, Posch T, Šimek K, Vrba J and 5 others (2001) Predator-specific enrichment of *Actinobacteria* from a cosmopolitan freshwater clade in mixed continuous culture. *Appl Environ Microbiol* 67:2145–2155
- Picard C, Ponsonnet C, Paget E, Nesme X, Simonet P (1992) Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl Environ Microbiol* 58:2717–2722
- Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. *Limnol Oceanogr* 25:943–948
- Reichenbach H (1992) The order *Cytophagales*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) *The Prokaryotes*. Springer, New York, p 3631–3675
- Riemann L, Winding A (2001) Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. *Microb Ecol* 42:274–285
- Riemann L, Steward G, Azam F (2000) Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl Environ Microbiol* 66:578–587
- Rosenstock B, Simon M (2001) Sources and sinks of dissolved free amino acids and protein in a large and deep mesotrophic lake. *Limnol Oceanogr* 46:644–654
- Schramm A, de Beer D, Wagner M, Amann R (1998) Identification and activities in situ of *Nitrospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. *Appl Environ Microbiol* 64:3480–3485
- Schweitzer B, Huber I, Amann R, Ludwig W, Simon M (2001) Alpha- and beta-*Proteobacteria* control the consumption and release of amino acids on lake snow aggregates. *Appl Environ Microbiol* 67:623–645
- Šimek K, Kojecká P, Nedoma J, Hartmann P, Vrba J (1999) Shifts in bacterial community composition associated with different microzooplankton size fractions in a eutrophic reservoir. *Limnol Oceanogr* 44:1643–1644
- Šimek K, Pernthaler J, Weinbauer M, Hornák K, Dolan J, Nedoma J, Mašin M, Amann R (2001) Changes in bacterial community composition and dynamics and viral mortality rates associated with enhanced flagellate grazing in a mesoeutrophic reservoir. *Appl Environ Microbiol* 67:2723–2733
- Simon M (1998) Bacterioplankton dynamics in a large mesotrophic lake: II. Concentrations and turnover of dissolved amino acids. *Arch Hydrobiol* 144:1–23
- Simon M, Bunte C, Schulz M, Weiss M, Wünsch C (1998a) Bacterioplankton dynamics in Lake Constance (Bodensee): substrate utilization, growth control, and long-term trends. *Arch Hydrobiol Spec Issues Adv Limnol* 53:195–221
- Simon M, Tilzer MM, Müller H (1998b) Bacterioplankton

- dynamics in a large mesotrophic lake: I. Abundance, growth and relationships to phytoplankton growth. *Arch Hydrobiol* 143:385–407
- Simon M, Glöckner FO, Amann R (1999) Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. *Aquat Microb Ecol* 18:275–284
- Simon M, Grossart HP, Schweitzer B, Ploug H (2002) Microbial ecology of organic aggregates in aquatic environments. *Aquat Microb Ecol* 28:175–211
- Southwick PL, Ernst LA, Tauriello EW, Parker SR, Mujumdar RB, Mujumdar SR, Clever HA, Waggoner AS (1990) Cyanine dye labeling reagents—carboxymethylindocyanine succinimidyl esters. *Cytometry* 11:418–430
- Spring S, Schulze R, Overmann J, Schleifer KH (2000) Identification and characterization of ecologically significant prokaryotes in the sediment of freshwater lakes: molecular and cultivation studies. *FEMS Microbiol Rev* 24: 573–590
- Stein LY, La Duc MT, Grundl TJ, Nealson KH (2001) Bacterial and archaeal populations associated with freshwater ferromanganese micronodules and sediments. *Environ Microbiol* 3:10–18
- Strunk O, Gross O, Reichel B, May M and 10 others (1998) ARB: a software environment for sequence data. Department of Microbiology, Technische Universität München, Munich (also available at <http://www.arb-home.de>)
- Teske A, Wawer C, Muyzer G, Ramsing NB (1996) Distribution of sulfate-reducing bacteria in a stratified Fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Appl Environ Microbiol* 62:1405–1415
- van Hanne E, Mooij W, van Agterveld MP, Gons HJ, Laanbroek HJ (1999) Detritus-dependent development of the microbial community in an experimental system: qualitative analysis by denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 65:2478–2484
- Wagner M, Amann R, Lemmer H, Schleifer KH (1993) Probing activated sludge with oligonucleotides specific for Proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Appl Environ Microbiol* 59:1520–1525
- Watson SW, Bock E, Valois FW, Waterbury JB, Schlosser U (1986) *Nitrospira-marina* gen. nov., sp. nov.: a chemolithotrophic nitrite-oxidizing bacterium. *Arch Microbiol* 144:1–7
- Wawer C, Jetten MSM, Muyzer G (1997) Genetic diversity and expression of the (NiFe) hydrogenase large-subunit gene of *Desulfovibrio* spp. in environmental samples. *Appl Environ Microbiol* 63:4360–4369
- Zwart G, Hiorns WD, Methé A, van Agterveld MP, Huismans R, Nold SC, Zehr JP, Laanbroek HJ (1998) Nearly identical 16S rRNA sequences recovered from lakes in North America and Europe indicate the existence of clades of globally distributed freshwater bacteria. *Syst Appl Microbiol* 21: 546–556
- Zwart G, Crump BC, Kamst-van Agterveld MP, Hagen F, Han SK (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquat Microb Ecol* 28:141–155

Editorial responsibility: Karel Šimek,
České Budějovice, Czech Republic

Submitted: August 13, 2001; Accepted: December 19, 2002
Proofs received from author(s): March 11, 2003