Seasonal Community and Population Dynamics of Pelagic Bacteria and Archaea in a High Mountain Lake

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The seasonal variations in community structure and cell morphology of pelagic procaryotes from a high mountain lake (Gossenköllesee, Austria) were studied by in situ hybridization with rRNA-targeted fluorescently labeled oligonucleotide probes (FISH) and image-analyzed microscopy. Compositional changes and biomass fluctuations within the assemblage were observed both in summer and beneath the winter ice cover and are discussed in the context of physicochemical and biotic parameters. Proteobacteria of the beta subclass (betaproteobacteria) formed a dominant fraction of the bacterioplankton (annual mean, 24% of the total counts), whereas alpha-proteobacteria were of similar relative importance only during spring (mean, 11%). Bacteria of the Cytophaga-Flavobacterium cluster, although less abundant, constituted the largest fraction of the filamentous morphotypes during most of the year, thus contributing significantly to the total microbial biomass. Successive peaks of threadlike and rod-shaped archaea were observed during autumn thermal mixing and the period of ice cover formation, respectively. A set of oligonucleotide probes targeted to single phylotypes was constructed from 16S rRNA-encoding gene clone sequences. Three distinct populations of uncultivated microbes, affiliated with the alpha- and beta-proteobacteria, were subsequently monitored by FISH. About one-quarter of all of the beta-proteobacteria (range, 6 to 53%) could be assigned to only two phylotypes. The bacterial populations studied were annually recurrent, seasonally variable, and vertically stratified, except during the periods of lake overturn. Their variability clearly exceeded the fluctuations of the total microbial assemblage, suggesting that the apparent stability of total bacterioplankton abundances may mask highly dynamic community fluctuations.

Until recently, microbial ecologist studying aquatic bacteria faced a basic dilemma: they could either measure the abundance, biomass, growth rates, activity, etc. of the “average” bacterium under in situ conditions (e.g., see reference 13), ignoring the phylogenetic and physiological diversity of microbial communities, or they could isolate and ecophysiological characterize individual bacterial strains (e.g., see reference 36) but were then not able to tell if these microorganisms were also common in the environment. Consequently, little knowledge has been gathered about the spatial and temporal abundance fluctuations of defined phylogenetic groups and of individual bacterial species in natural habitats. Molecular biological techniques used to identify microbes in environmental samples have recently provided new tools to study bacterioplankton biodiversity (e.g., see references 1, 9, 14, 15, and 19) and the in situ abundances of bacteria and archaea that could not be adequately distinguished before (2, 4, 5, 25). Microbiologists are now in a position to potentially elucidate the biogeography (24), population dynamics, and successions (28) not only of a few morphologically conspicuous microbes but of a large number of species, most of which might still be uncharacterized.

Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes selectively visualizes bacterial cells with defined phylogenetic affiliations (3, 5). Based on a rapidly growing set of 16S (and, to a lesser extend, 23S) rRNA sequence data, it is probably the phylogenetically most sophisticated (22) approach for whole-cell in situ identification. On the other hand, FISH of plankton samples can be performed with minimal laboratory requirements (16), and evaluation relies on epifluorescence microscopy, which is a standard technique of aquatic microbial ecologists, e.g., for counting (30) and sizing (33) of picoplankton. In contrast to other identification approaches, FISH largely conserves the gestalt of the targeted microorganisms, i.e., their morphologies, cell sizes (26, 34), and cellular rRNA content (7, 32). So, despite the limitations of the method (as discussed in reference 5), its potential for the identification and cytometric analysis of planktonic microbes is just about to be recognized.

Recent investigations have reported that various freshwater microbial communities are dominated by bacteria which are phylogenetically affiliated with the alpha and beta subclasses of the class Proteobacteria (alpha- and beta-proteobacteria, respectively) and with members of the Cytophaga-Flavobacterium cluster (2, 6, 16, 19). These observations were based on single or short-term sampling schemes. The instantaneous community composition of the bacterioplankton, however, may not be representative for different seasons, and the typical ranges of annual community variability remain to be established.

The size distribution of planktonic bacteria, and particularly the appearance of filamentous cells, has come into the focus of aquatic microbial ecology in the context of studies of predator-prey interactions. It has been shown both in the laboratory (18, 37) and in field experiments (20) that the filamentous morphotype is a phenotypic adaptation of some microbes to protistan grazing, but there are probably numerous other causes for bacteria to elongate far beyond their typical sizes (e.g., see reference 23). Threadlike bacteria have been observed throughout the year in the plank-
ton of a hypertrophic lake (41) but were also found in midwinter in an oligotrophic alpine lake (31).

In earlier studies, we demonstrated FISH to be an appropriate tool for the monitoring of spatial (2) and short-term temporal (26) dynamics of different phylogenetic groups of the planktonic microbial community in a high mountain lake. Here we report on the seasonal and vertical abundance distributions of pelagic members of Bacteria and Archaea in Gossenköllesee and analysis of the community structure at different levels of taxonomic resolution. We applied published domain- and group-specific oligonucleotide probes (5) but also used the sequence information from a 16S rRNA-encoding gene (rDNA) library obtained from Gossenköllesee bacterioplankton 1 year earlier to construct specific probes targeted at individual bacterial populations. Particular attention was paid to the changes in abundance and taxonomic composition of the filamentous bacterial morphotypes which were recognized as a permanently important fraction of the planktonic procytotes in Gossenköllesee. Additionally, we monitored the seasonal changes in the biomass size distributions of the nonfilamentous fraction of the pelagic microbial community.

MATERIALS AND METHODS

Sampling site and procedure. The study site, Gossenköllesee, is a small, oligotrophic high-mountain lake in the Central Alps (Tyrol, Austria), situated above the timberline at an altitude of 2,417 m (11). The catchment area of Gossenköllesee is listed as a United Nations Educational, Scientific, and Cultural Organization Man and Biosphere Reserve. Annual water temperatures typically range between 0 and 15°C, and thermal mixing occurs from late October to mid- November and from late June to early July (44). During the summer months, the lake is exposed to high levels of solar UV radiation (40). The lake was ice covered from mid-November 1996 until the end of June 1997, and during this period, dissolved oxygen decreased to well below saturation in the lower water layers (44). Table 1 depicts the development of temperature, dissolved oxygen, conductivity, and the chlorophyll a concentration during the study period (from reference 44).

Water samples from three depth layers (the surface, 4 m, and 8 m) were collected monthly between 4 July 1996 and 25 June 1997 with a 5-liter Schindler-catcher (from reference 44). This period, dissolved oxygen decreased to well below saturation in the lower water layers (41). During the summer months, the lake is exposed to high levels of solar UV radiation (40). The lake was ice covered from mid-November 1996 until the end of June 1997, and during this period, dissolved oxygen decreased to well below saturation in the lower water layers (44). Table 1 depicts the development of temperature, dissolved oxygen, conductivity, and the chlorophyll a concentration during the study period (from reference 44).

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<table>
<thead>
<tr>
<th>Date</th>
<th>Temp (°C)</th>
<th>O₂ (% saturation)</th>
<th>Alkalinity (µEq liter⁻¹)</th>
<th>Chlorophyll a concn (µg liter⁻¹)</th>
</tr>
</thead>
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<td></td>
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<td>8 m</td>
<td>Surface</td>
</tr>
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<td>4.1</td>
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<td>7.6</td>
<td>5.9</td>
<td>5.8</td>
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</tbody>
</table>

* Adapted from reference 44.

Design and characterization of oligonucleotide probes. Water samples for DNA extraction were taken at Gossenköllesee on 11 December 1995 from a depth of 3 m. Around 900 ml of 50-µm-prescreened lake water was filtered with hydrophilic filters (Durapore [pore size, 0.2 µm; diameter, 47 mm]; Millipore Corp., Bedford, Mass.) until the filters were completely clogged with plankton biomass. During filtration, the samples were kept at ambient water temperatures (2 to 3°C). The filters were cut into smaller sections and stored at −20°C until further processing. DNA extraction from the filters was performed by following the protocol of Fuhrman et al. (14). Almost full-length bacterial 16S rDNA fragments were amplified by PCR from the extracted DNA by using two general bacterial 16S rDNA primers (38) on a Hybid OmniGene thermocycler (MWG-Biotech, Ebersberg, Germany). Amplification conditions were 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min for 30 cycles after an initial preheating step of 94°C for 3 min. PCR amplification products were purified and cloned as described previously (38). Fifty 16S rDNA clones were partially sequenced (300 to 500 nucleotides) by using a LICOR DNA 4000 automated sequencer (MWG-Biotech) (38). These partial sequences were added to the 16S rDNA sequence database of the Technical University of Munich with the program package ARB (43). Two clones each affiliated with the alpha (GKS59 and GKS69) and beta (GKS16 and GKS98) proteobacteria were selected, and their 16S rDNA inserts were fully sequenced. The clone identifications, EMBL accession numbers, and nearest relatives in the database are presented in Table 2. By using the expected PROBE DESIGN tool of ARB (43), specific oligonucleotide probes were constructed. In order to minimize the risk of accidentally detecting more than one population by FISH, we designed two oligonucleotide probes per clone that were targeted at different positions on the 16S rDNA and each of which showed at least one mismatch with all other known sequences. All probes were purchased from Interactiva (Ulm, Germany) and labeled with the indocarboxyanine dye Cy3. The sequences and target positions of the clone-specific probes are given in Table 2.

The sequencing of the functional and structural genes (16S rDNA and 23S rDNA, respectively) of each of the 16S rDNA clones was performed on an Applied Biosystems 373A DNA sequencer at the Max Planck-Institut für Molecular Physik, Berlin, Germany.

The sequences were analyzed and the phylogenetic affiliations were assigned by using the programs PHYLIP (10) and the domain bacterial 16S rRNA sequence database of the National Center for Biotechnology Information (NCBI) at GenBank (http://www.ncbi.nlm.nih.gov/Genbank/).

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were separated by distinct minima in July, October, and February. Two peaks of total numbers in early autumn and midwinter were detected. Accordingly, only the relative fractions of filamentous archaea are depicted in the results (see Fig. 2).

**RESULTS**

**Total assemblage.** Cell densities determined by DAPI staining ranged from $0.11 \times 10^5$ to $0.55 \times 10^5$ cells ml$^{-1}$ (Table 3). Two peaks of total numbers in early autumn and midwinter were separated by distinct minima in July, October, and February. Filamentous cells were present in the plankton throughout the season at densities of $0.076 \times 10^3$ to $1.33 \times 10^5$ ml$^{-1}$, reaching maximal abundances in early autumn and continuously decreasing in number during the period of ice coverage.

In early winter, filaments were more frequent in the lower water layers. Three clearly separated maxima of nonfilamentous bacterial biomass were found in late summer, midwinter, and spring (Fig. 1). Between July and January, a considerable amount (>35% in September and October) of biomass was present in cells >3.2 μm in length, and during August and September, the biomass was distributed more evenly over bacterial size classes. Between March and June, biomass maxima were formed exclusively by small bacteria (0.4 to 1.2 μm) and were more pronounced in the deeper water layers.

**FISH with domain- and group-specific probes.** Between 26 and 82% of all DAPI-stained objects (mean, 55%) could be visualized with bacterial probe EUB338, and between 68 and 100% of filamentous cells (mean, 87%) were detected by EUB338 (Table 3). Detection rates were lowest at a depth of 8 m (mean, 53%) between December and February for all cells and from October to December for filamentous forms. Archaea represented from less than 1% to a maximum of 5% of all DAPI-stained cells (mean, 1.4%) and reached their highest densities in September and shortly after ice cover formation in late November (Fig. 2). During September and October, there was a distinct maximum of the filamentous fraction of archaea throughout the water column. On an annual average, beta-proteobacteria formed 24% (range, 5 to 41%) of all DAPI-stained cells and 45% (range, 20 to 89%) of the cells detected by the bacterial probe EUB338.

**TABLE 3. Total picoplankton and filament abundances in Gossenköllesee and detection by probe EUB338**

<table>
<thead>
<tr>
<th>Date</th>
<th>Total cell conc ($10^5$ ml$^{-1}$)</th>
<th>% detected by EUB338</th>
<th>Filament conc ($10^3$ ml$^{-1}$)</th>
<th>% detected by EUB338</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Surface 4 m 8 m</td>
<td></td>
<td>Surface 4 m 8 m</td>
<td></td>
</tr>
<tr>
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<td>2.07</td>
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</tr>
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</tr>
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<td>3.45</td>
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<td>1.85</td>
<td>2.15</td>
<td>1.93</td>
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</table>

* Percentage of cells stained by DAPI.
At all three depths, the beta-proteobacteria reached their maximal abundances in late summer (August to September) (Fig. 3a). Another maximum during midwinter (December and January) was followed by a sharp decline in numbers. The alpha-proteobacteria showed a similar peak in early autumn in the upper water layers (Fig. 3b). A second peak was observed in May at a depth of 4 m, succeeding an annual maximum of chlorophyll $a$ which occurred at the same depth during early spring (Table 1). Alpha-proteobacteria accounted for 4 to 23% of all DAPI-stained cells (mean, 11%) and, on average, 21% of the EUB338 counts (range, 7 to 45%).

The ratio of alpha- to beta-proteobacteria ranged from 0.15 in midwinter and early summer to 0.90 during April and May (annual mean, 0.50), with distinct maxima in autumn (September to November) and spring (March to May) at the surface and at a depth of 4 m. Gamma-proteobacteria always accounted for less than 2% of the total cell density (data not shown).

Bacteria of the *Cytophaga-Flavobacterium* cluster never exceeded $4 \times 10^4$ ml or 10% of all DAPI-stained cells (mean, 3.5%) (Fig. 3c). Threadlike morphotypes formed up to 80% of all of the bacteria hybridizing with CF319a in the surface layer during October but were virtually undetectable during early summer (June and July; annual mean, 20%) (Fig. 4). Members of this phylogenetic cluster were a dominant fraction of all filamentous bacteria for most of the year (mean, 53%), except for two distinct minima in summer and winter, respectively.

**Populations monitored with clone-specific probes.** Each pair of probes was found to define identical populations, i.e., two probes targeted at different positions of one rRNA sequence (Table 2) detected bacterial cells that could not be distinguished in terms of their numbers in parallel samples and their cell morphologies. As the pairs of probes appeared to be equivalent in terms of detected population sizes, evaluation of the
seasonal samples was performed by using the probe that gave the brighter hybridization signal (probes GKS16-442, GKS69-218, and GKS98-1459). The hybridization of RNA transcripts from the 16S rDNAs with these probes at various concentrations of formamide resulted in typical sigmoidal melting curves (Fig. 5). Probes targeted to three of the four clones detected bacterial populations that at least seasonally exceeded 1% of the DAPI-stained cells (Fig. 6a to c). The sequence of the fourth clone, GKS59, affiliated with alpha-proteobacteria, was associated with a filamentous morphotype (cell length, 11 to 20 \( \mu m \)) that occurred sporadically throughout the year at low densities (data not shown). The bacteria hybridizing with probes GKS16-61 and GKS16-442 were rod-shaped cells (lengths, 1.5 to 3.5 \( \mu m \)) and short filaments (8 to 13 \( \mu m \)). The phylotype GKS16 is affiliated with beta-proteobacteria, and *Rhodoferax fermentans* is its closest known relative with a 16S rRNA similarity of 93.9%. These populations were most abundant between September and January, and the populations were seasonally more stable at the surface than in the lower water layers (Fig. 6a). The maximal relative densities were 6% of DAPI-stained cells. At 4 m, we observed a biphasic annual population development: a distinct spring bloom between March and May was set apart from the previous autumn to early winter maximum by a decline to a level below the detection limit during February. A similar drop was also observed at a depth of 8 m. On both dates in July, when the lake mixes, population densities were very similar at all three depths.

The second beta-proteobacterial population hybridizing with GKS98-826 and GKS98-1459 consisted of rods with cell lengths of 2 to 4.5 \( \mu m \). They showed a clearly parallel seasonal development at all depths (Fig. 6b). After a decline below the detection densities in August, the populations reached maximal abundances during the period of ice layer formation (4% of DAPI-stained objects) and then declined below the limit of detection during the early spring. On a seasonal average, cells hybridizing with probes for clones GKS16 and GKS98 constituted about one-quarter of the beta-proteobacteria, but this fraction fluctuated between >50% in the surface layer during the formation of the winter ice cover and less than 10% in the lower water layers during February.

Bacteria targeted by probe GKS69-218 or GKS69-1451 were brightly fluorescent rods with cell lengths of 2.8 to 9.5 \( \mu m \). The phylotype GKS69 clusters with the genus *Sphingomonas* within the alpha-proteobacteria. The respective population appeared sporadically at densities of 5,000 to 10,000 cells ml\(^{-1}\) between August and March, mainly in the upper water layers (Fig. 6c).

**DISCUSSION**

Seasonal community changes. Bacterial in situ identification and the determination of total biomasses appear to describe supplementary features of microbial communities. Either analysis established that the microbial assemblage of Gossenköllesee continuously transformed, even during the months when the lake was sealed by the winter ice cover. The icebreak period between June and July appeared to be a major promoter of community transition (c.f. reference 26). This is easily conceivable by considering, e.g., the allochthonous input from the winter ice cover (35a), the subsequent thermal mixing of the water body (Table 1) and the increased exposition to sunlight. Recently, Sommaruga and coworkers demonstrated that UV A and B radiation readily penetrates the water column of Gossenköllesee and that this is sufficient to directly and indirectly affect bacterial \(^3^H\)thymidine and \(^{14}C\)leucine incorporation.
with the bacterial probe dropped (Table 3), the numbers of beta-proteobacteria and filamentous members of the **Cytophaga-Flavobacterium** cluster declined significantly (Fig. 3a and 4), and bacteria related to clone GKS16 dropped below the limit of detection in all but the surface water layers (Fig. 6a). Simultaneously, the biomass of very small bacteria increased (Fig. 1), concomitant with the winter maxima of chlorophyll a concentrations (Table 1). The presence of an active microbial community beneath the winter ice cover was also indicated by the gradual decline of oxygen saturation in the lowest water layers (Table 1 in reference 44). The pelagic bacteria in Gossenköllesee formed a second biomass maximum in late spring in the lower water layers (Fig. 1), in parallel with a density increase of both the alpha- and beta-proteobacteria (Fig. 3a and b) and of the population hybridizing with GKS16-442 (Fig. 6a). This development coincided with the decline of the phytoplankton bloom at a depth of 4 m (Table 1) but also with the onset of the snowmelt in the catchment area (44), as reflected by decreased lake water alkalinity at 0 m (Table 1). This water influx might be the cause for the reduced bacterial biomass at the lake surface prior to the ice break (Fig. 1).

**Bacteria of the Cytophaga-Flavobacterium cluster** bloomed in deeper water layers in summer, at the surface during ice formation, and at the depth with the highest chlorophyll a concentrations in early spring (Table 1). In summary, this hints at phylogenetic diversity within this group masked by the generality of the probe. This conclusion is also supported by the contrasting dynamics of the filamentous and nonfilamentous morphotypes (Fig. 3c and 4). A fraction of these threadlike bacteria might indeed genotypically resemble small cells with a high potential for morphological plasticity, as has been shown, e.g., for marine psychrophiles (23) and a *Comamonas acidovorans* strain isolated from a lake (18). Since a prominent part of the planktonic filaments in Gossenköllesee may reach cell lengths of >50 μm (31), they form a considerable fraction of the total microbial biomass. During extended periods, most of the threadlike bacteria were affiliated with the *Cytophaga-Flavobacterium* cluster (Fig. 4), so that this phylogenetic group at least seasonally constituted the largest pool of bacterial biomass in the system.

**Studying microbial population dynamics by FISH.** It has been shown that releasing allochthonous microbes into the plankton may result in their rapid disappearance (see, e.g., reference 7), but the fate and dynamics of autochthonous bacterioplankton populations is much less well understood. Gordon and Giovannoni (17) monitored the seasonal density variation of a bacterial rDNA sequence in the Atlantic; yet they did not attempt to estimate absolute cell abundances. Recently, Pinhassi et al. (28) presented a protocol to determine the in situ densities of individual bacterial species which, however, requires prior cultivation of the target organisms. Our approach differs from these in that it analyzes single bacterial cells rather than nucleic acid extracts. Thus, we could take into account the microscopically obvious difference between filamentous and nonfilamentous bacteria, which is certainly of ecological relevance, e.g., considering growth rates (18) or biomass contribution to the community. Recently, FISH has been successfully applied in Antarctic coastal water (25). A similar but indirectly also cultivation-dependent strategy used to monitor bacterial populations in a lake based on fluorescently labeled monoclonal antibodies has been described by Faude and Höfle (10). The two populations investigated in that study together formed less than 0.1% of the total bacterial community, which demonstrates the difficulty of specifically isolating those microbes from the plankton that form larger populations in situ.

Probes designed for single retrieved 16S rRNA sequences of uncultivated microorganisms cannot be easily tested for specific-
ity. We used pairs of probes targeted to distant positions of the 16S rRNA and compared abundances and morphologies of hybridized cells from parallel samples in order to minimize the risk of detecting multiple populations. In addition, melting curve equivalents were determined by hybridizing the sequence-specific probes against RNA transcripts from the retrieved 16S rDNAs (Fig. 5), which have been shown to resemble the melting behavior of native rRNA (29). On these grounds, it was possible to set hybridization conditions that ensured the specificity of the probes for the respective rRNA targets. Yet, for definitive confirmation of the identity of the targeted 16S rDNA (and not just the target region), it might be necessary to, e.g., flow sort the respective microbes and then amplify and sequence their 16S IDNAs (45).

Knowing that some bacterial species might have practically identical 16S rDNA sequences (12), can one actually study the population dynamics of a single bacterial species by in situ hybridization? Considering macroecological principles of competitive exclusion, it is probably unlikely to find closely related microorganisms co-occurring in a nutrient-poor system dominated by strong physical and chemical fluctuations. One must, however, be aware of the physiological variability that may be found within different DNA-DNA homology groups of one single species (35). We thus caution against premature conclusions about the ecology of microorganisms based exclusively on their in situ occurrence if not backed up by taxonomic and eco-physiological studies on isolates.

However, the knowledge of in situ distributions of microbial populations at our level of analysis provides a basis for such research, as it allows us to set the pelagic bacteria into the traditional framework of plankton ecology. By applying FISH for physiological studies on isolates.

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Knowing that some bacterial species might have practically identical 16S rDNA sequences (12), can one actually study the population dynamics of a single bacterial species by in situ hybridization? Considering macroecological principles of competitive exclusion, it is probably unlikely to find closely related microorganisms co-occurring in a nutrient-poor system dominated by strong physical and chemical fluctuations. One must, however, be aware of the physiological variability that may be found within different DNA-DNA homology groups of one single species (35). We thus caution against premature conclusions about the ecology of microorganisms based exclusively on their in situ occurrence if not backed up by taxonomic and eco-physiological studies on isolates.

However, the knowledge of in situ distributions of microbial populations at our level of analysis provides a basis for such research, as it allows us to set the pelagic bacteria into the traditional framework of plankton ecology. By applying FISH for physiological studies on isolates.

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