A transplant experiment to identify the factors controlling bacterial abundance, activity, production, and community composition in a eutrophic canyon-shaped reservoir

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Abstract

We performed a transplant experiment in eutrophic Sau reservoir to assess the factors that control bacterial abundance, activity, growth rate, and community composition. Samples from the lacustrine and the riverine ends of the reservoir were incubated in dialysis bags placed in situ and transplanted to the other side of the reservoir and also incubated after 1 μm filtration to measure predator effects. The bags were sampled at 12-h intervals to estimate bacterial abundance, whole community activity, activity structure (by flow cytometry), and phylogenetic composition (by in situ hybridization with group-specific phylogenetic probes). Bacterial production was always regulated by nutrient supply, but abundance and activity were differently regulated at both sites. The riverine bacteria were limited by predator activity, whereas the lacustrine were regulated by a combination of predation and nutrient supply. Therefore, even in the same environment, different modes of control can act simultaneously. Bacterial activity structure was also regulated in the same way. Abundance of highDNA bacteria and cells hybridizing with the universal EUB338 probe were well correlated. In the lacustrine sample, bacterial community structure did not change significantly, whereas in the riverine sample, α- and γ-Proteobacteria reduced their growth when transplanted, whereas β-Proteobacteria were stimulated by the presence of predators. Members of the Cytophaga/Flavobacterium phylum grew only when incubated in situ in the absence of predators. This different behavior in the different bacterial groups resulted in strong changes in bacterial assemblage composition, evident already after 24 h. The experiment demonstrates that, together with the effect of predators, nutrient supply affects bacterial community properties and that a complex regulation involving both types of control can occur in a single heterogeneous planktonic system.

Bacteria are relevant members of the limnetic planktonic food web, both in terms of biomass and production share (e.g., Cole and Caraco 1993; del Giorgio and Gasol 1995) and while this is very evident in oligotrophic planktonic systems, it is also true for eutrophic environments (Sommaruga and Robarts 1997). The assessment and comprehension of the factors that control bacterial abundance and production will facilitate the understanding of how carbon and nutrients circulate in planktonic food webs. Improved models and more successful manipulations will be achieved with better understanding of these factors.

It is commonly believed that the actual amount of cells (abundance) and the rates of activity and production are either determined by the available nutrient concentrations (so-called bottom-up control) or by the effect of predators (so-called top-down control). Bacterial production is considered to be controlled by the rate of nutrient supply, and final abundances and the rates of specific growth are considered to be determined by predation pressure, by substrate supply, or by both (Wright and Coffin 1984; Pace and Cole 1994; Thingstad and Lignell 1997). Viruses seem to be more important in regulating community diversity (Thingstad and...
Lignell 1997) or the rate of total respiration. Thus, more nutrients would mean more biomass and production, and more predators could mean less biomass or activity. This simple framework has been routinely used to infer the main mode of control of the different microbial populations in whole-system manipulations (Pace and Cole 1996) and empirical analysis of databases (Sanders et al. 1992; Pace and Cole 1994), but no clear-cut conclusions have been reached yet, in part because of the impossibilities of replicating naturally modified nutrient supply rates.

We now know, furthermore, that nutrient availability and predation control can not only regulate abundances and activities but also specific activities and growth rates and bacterial community composition (BCC). We know, for example, that predators can alter bacterial size distribution by preying on the largest cells (e.g., Jürgens and Güde 1994), specific activity by preying on the most active cells (del Giorgio et al. 1996), and BCC by preferentially preying on given groups of cells (Šimek et al. 1997; Jürgens et al. 1999; Suzuki 1999).

During the past few years, we have come to learn quite a lot about BCC in freshwater environments with in situ hybridization with oligonucleotide rRNA-targeted probes. Data are now available from oligotrophic (Alfreider et al. 1996; Pernthalter et al. 1997), oligomesotrophic (Glöckner et al. 1996; Methé et al. 1998), and eutrophic lakes (Casamayor et al. 2000) and reservoirs (Šimek et al. 1999). And although experimental evidence of the role of in situ manipulating predation pressure on BCC exists (Jürgens et al. 1999; Šimek et al. 1999), nutrient availability is not commonly manipulated (but see Fisher et al. 2000, for an exception). Instead, evidences of the effect of nutrient supply are indirect, based on following BCC before, during, and after an algal bloom (e.g., Riemann et al. 2000).

Because of their relatively short retention times and longitudinal homogeneities, canyon-shaped narrow and long reservoirs are spatially heterogeneous systems with extremely pronounced longitudinal gradients in diversity and functioning of microbial food webs (Comerma et al. 2001; Šimek et al. 2001) from the riverine to the lacustrine parts of the reservoir that had very different characteristics (see below), because of the inputs of large amounts of nutrients and organic matter in its inflow side (Armengol et al. 1999; Comerma et al. 2001). Water from both sites was collected in the early morning of 12 May 1998 and transported in the dark in Nalgene containers to the nearby laboratory. There, half the volume of each sample was filtered at low pressure through 1-µm Nuclepore filters, yielding likely bacterivore-free treatments. Immediately afterward, both the filtered and unfiltered water from both sites were placed in 24 dialysis bags each. The dialysis bags (Sigma), with a cutoff size of 6,000–8,000 Da and a maximal width of ~8 cm, were cut in lengths of 20–25 cm to hold 400–500 ml. The bags had been thoroughly washed in hot tap water, rinsed overnight, and then soaked for >3 h in Milli-Q water before use. After they were filled, they were closed and attached to the inner part of small wired cages. We placed two cages, one in the riverine part of the reservoir at Sta. 9 (from now on called “River”) and another one in the lacustrine part of the reservoir at Sta. 1 (from now on called “Dam”). In each box we tied 24 dialysis bags: 6 replicates of untreated sample (Dam water in the Dam site and river water in the River site), 6 of transplanted sample (Dam water in the River site and river water in the Dam site), 6 of filtered sample, and 6 more of filtered and transplanted sample. The boxes were attached to weights and submerged at ~0.5 m from the surface at the relevant positions in the reservoir.

We had, thus, two water samples and four treatments (control, CTL; transplanted, TR; bacterivore-free, BF; and bacterivore-free and transplanted, BF + T). Two dialysis bags of each treatment were removed at ~12 h, two more at ~24 h, and the last ones at 48 h. They were immediately brought to the laboratory and processed as described below. The incubation was started around noon on 12 May. The second sample was taken around midnight and the other two samples around noon on 13 and 14 May. Thus, from 0 to 12 h corresponds to daylight samples, and from 12 to 24 h corresponds mainly to night samples. Although bacterial abundances and activities were measured in all replicates and in all times, phylogenetic community composition was ana-
lyzed only at times 0, 24, and 48 h and in one replicated bag only. In previous experience with similar experimental setups, we have observed BCC in replicated bags to vary with coefficients of variation <15% (e.g., Šimek et al. 1999 and unpubl. data). Predators were only counted in the unfiltered samples, but it was repeatedly confirmed at different experimental times that they were absent in the BF <1 μm, treatment.

Two-way ANOVAs, with Bonferroni-corrected post hoc tests of significance that used the data of times >12 h were used as a method to test for the effects of each treatment. Data of the growth rate estimates, which had no replication, and protozoan abundances, which were measured only in two treatments, were analyzed with t tests.

Bacterial abundance and biomass—A 1.2-ml subsample for bacterial counts was preserved with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration), frozen in liquid nitrogen, and stored in a −70°C freezer to determine bacterial abundance and relative size by flow cytometry. The samples were later unfrozen, stained for a few minutes with Syto13 (Molecular Probes) at 2.5 μM, and run through a flow cytometer. We used a Becton & Dickinson FACScalibur bench machine with a laser emitting at 488 nm. Samples were run at low speed (~18 μl min⁻¹), and data were acquired in log mode until around 10000 events had been recorded. We added 10 μl per sample of a 10⁶ μl⁻¹ solution of yellow-green 1 μl Polysciences latex beads as an internal standard. The beads were calibrated against epifluorescence counts or against B&D TruCount beads of a known concentration. Bacteria were detected by their signature in a plot of side scatter versus green fluorescence as explained in Gasol and del Giorgio (2000). The fixed samples were diluted 2×–4× with Milli-Q water so that the rate of particle passage was kept below 500 particles per second, and, thus, coincidence was avoided (Gasol and del Giorgio 2000). The average fluorescence of the bacterial population, as normalized to that of the beads, is a rough approximation of bacterial size, although in such a eutrophic environment, with most bacteria being large and filamentous, average fluorescence underestimates size.

Bacterial activity and production—We estimated bacterial activity from radioactive Leucine (Leu) incorporation using standard methods and in Eppendorf vials. We added 40 nM Leu to sextuplicate vials. That was a saturating concentration, as found in two concentration-dependent incorporation experiments performed at both sides of the reservoir. We used two 50% trichloroacetic acid-killed samples as controls and four experimental replicates. The vials were incubated in the dark and in temperatures as close as possible to the original ones for 45–60 min. Bacterial heterotrophic production was calculated as Leu incorporation rate times the standard 3.1 kgC mol Leucine⁻¹ conversion factor. This factor assumes a twofold dilution of the exogenously added Leu with the autochthonous leucine. In a very eutrophic environment, this is not necessarily true, and we would expect bacterial production to be underestimated with this protocol. From the estimates of bacterial production (BHP) and those of bacterial biomass (BBM), we obtained specific growth rates (SGR) and doubling times (Td) as

\[
SGR = \ln(1 + \text{BHP}/\text{BBM}), \quad Td = \ln 2/\text{SGR}
\]

Bacterial production was also measured via thymidine (TdR) incorporation. Five 5-ml subsamples were incubated for 30 min at in situ temperature with 20 nM of [methyl⁻³H] TdR (DUPON Corp.), then preserved with neutral buffered formaldehyde (2% final concentration), filtered through 0.2-μm polycarbonate filters (Poretics), and extracted four times with 1 ml of ice-cold 5% trichloroacetic acid by five washes of 1 ml of ice-cold 80% ethanol. Replicate blanks prefixed by 2% formaldehyde were processed in parallel. Three different empirical conversion factors, determined in May 1998 (Comerma et al. 2001), were used, one for the Dam area stations, a second for the central stations, and a third for the riverine stations.

Bacterial community composition—Three approaches were used for determining bacterial community composition: (1) simple cytometrically derived community composition, (2) direct DNA analysis by fingerprinting denaturing gradient gel electrophoresis (DGGE), and (3) fluorescent in situ hybridization (FISH).

(1) We used the percentage of high DNA bacteria (%HDNA) as a simple way of describing the activity structure of the bacterial community. The observation of Syto13-stained bacteria in the flow cytometer allows the clear separation of two subgroups, which we have called HighDNA and LowDNA bacteria (Gasol et al. 1999; Gasol and del Giorgio 2000). A growing body of evidence suggests that the HighDNA bacteria are the active members of the community (Gasol et al. 1999; Servais et al. 1999; Lebaron et al. 2001), whereas the LowDNA bacteria include the “bacterial ghosts” and other inactive bacteria (Gasol et al. 1999).

(2) For fingerprinting comparison, plastic containers were filled up with 5 L of water and kept in the dark on ice until processing in the laboratory a few hours later. Cells were concentrated by use of a refrigerated centrifuge at 8,000 × g and kept frozen at −70°C until further use. Microscopic observation of the supernatants showed that between 1% and 3% of the cells were not recovered by this method. DNA was extracted with hot sodium dodecyl sulfate/phenol and phenol-chloroform-isoamylalcohol followed by ethanol precipitation. DNA was used as target in the polymerase chain reaction (PCR) to amplify the bacterial 16S rRNA genes with the primer combination 341F with GC clamp (40 nucleotide GC-rich sequence 5'-CCT AGC GGA GGC AGC AG-3') and 907R (5'-CCG TCA ATT CMT TGT AGT TT-3'). PCR conditions and DGGE were as described elsewhere (Casamayor et al. 2000). The DGGE gel was stained with ethidium bromide and photographed under ultraviolet light (314 nm) by use of a Polaroid camera. The DGGE bands were excised from the gels, reamplified, and sequenced as reported (Casamayor et al. 2000). They were then submitted to BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/), to determine the closest relatives in the database. They were aligned by use of the ARB program package (http://www.mikro.biologie.tu-muenchen.de) and the percentage similarity calculated. The sequences were deposited at the
EMBL (European Molecular Biology Laboratory) Nucleotide Sequence Database under the accession numbers AJ311502–AJ311507.

(3) A gross analysis of bacterial community structure was carried out by FISH with group-specific RNA-targeted oligonucleotide probes on membrane filters (Alfreider et al. 1996; Glöckner et al. 1996). Duplicated samples were prefixed with alkaline Lugol’s solution followed by formaldehyde (2% w/v, final concentration) for at least 1 h and decolored by addition of several drops of a 3% solution of sodium thiosulphate, to prevent cell disruption of algae and other fragile flagellates. Bacterial cells from 10- to 20-ml subsamples were concentrated on white 0.2-μm pore filters (Poretics Corp.; 47-mm diameter), rinsed with distilled water, and stored frozen at −20°C until further processing. FISH of filter sections with the five different oligonucleotide probes targeted to the kingdom Bacteria (EUB), the phyla α-, β-, γ-subclasses of Proteobacteria (ALF, BET, and GAM), and to the Cytophaga/Flavobacterium group (CF) was carried out as described in Alfreider et al. (1996). The probes were fluorescently labeled with the indocarbocyanine dye Cy3 (Interactiva, Ulm, Germany). After hybridization, the filter sections were stained with 4’,6-diamino-2-phenylindole (DAPI), and the percentage of hybridized bacterial cells enumerated by epifluorescence microscopy (PROVIS AX 70, Olympus). At least 500 DAPI-stained cells per sample were inspected. To significantly reduce fading of the probe signal, the mounting medium Citifluor (Citifluor Ltd., Kent, UK) was supplemented with VectaShield (Vector Laboratories, Burlingame, CA; Pernthaler pers. comm.) at 80%: 20%, respectively.

**Protists and grazing impact**—Thirty-milliliter subsamples for protzoan enumeration were taken and fixed by adding 0.5% of alkaline Lugol’s solution, immediately followed by 2% borate-buffered formaldehyde (final concentration) and several drops of 3% sodium thiosulfate to clear the Lugol’s color. Five milliliter (flagellates) or 20–30 ml (ciliates) subsamples were stained with DAPI, filtered through 1-μm (flagellates) or 2-μm (ciliates) black Poretics filters, and inspected via epifluorescence microscopy. Nonpigmented, heterotrophic nanoflagellates (HNF) and plastidic flagellates were always differentiated. We estimated protzoan grazing on bacterioplankton from past measurements of protzoan
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Fig. 2. Variation of some bacterial parameters along the reservoir on 12–14 May 1998. (A) Total, HighDNA, and LowDNA bacteria. (B) %HDNA and average bacterial green fluorescence, as related to that of 1-μm Polysciences beads in a flow cytometer. (C) Leu and TdR incorporation rates. (D) Total bacterial growth rates calculated from the TdR-based (0–2 scale) and from the Leu-based (0–0.16 scale) bacterial production and biomass duplication time (Dt), based on the TdR data. The vertical arrows indicate the sources of water for the transplant experiment and the situation in which they were incubated.

grazing on fluorescently-labeled bacteria (FLB) on Sau (Šimek et al. 2000, 2001). For HNF, the average value from 16 measurements performed in Sau was 16.3 bact HNF⁻¹ h⁻¹. For ciliates, we used the species-specific grazing rate calculated elsewhere (Šimek et al. 2000, averages 630–2,000 bact cil⁻¹ h⁻¹) and combined these data to the actual ciliate composition in the Sau samples.

Results

The canyon-like shape of the Sau reservoir generates a very clear longitudinal heterogeneity in limnological characteristics from the riverine to the lacustrine ends (Armengol et al. 1999). In May 1998, the river was rich in total phosphorus (~6 μM) and nitrogen (~200 μM) and had characteristic high levels of soluble reactive phosphorus (SRP; ~3 μM) and ammonia (~175 μM) and low levels of nitrate (~20 μM). In contrast (Fig. 1), the water at the Dam side had lower total phosphorus (~2 μM) and nitrogen (~140 μM) and characteristically higher nitrate (~3 μM) and lower SRP (~0.5 μM) and ammonia (~50 μM). Accompanying these physical changes, total chlorophyll a decreased from values of >30 mg m⁻³ in the riverine parts to values of 15 mg m⁻³ in the Dam area (Fig. 1). Even though bacteria were more concentrated (Fig. 2) in the river side (~1.2 × 10⁷ ml⁻¹) than in the Dam side (~4 × 10⁶ ml⁻¹), a clear decrease in bacterial abundance occurred as soon as the river entered the lacustrine part of the reservoir; thus, bacterial abundance at 14 km from the Dam (the site used for the experiment) was already slightly lower than that at the Dam site. Major changes in bacterial concentration occurred between 8 and 14 km from the Dam, as has been reported elsewhere (Comerma et al. 2001; Šimek et al. 2001), but we did not use these samples for the experiment. More conspicuous than the changes in bacterial abundance were the changes in the relative proportion of HighDNA and LowDNA bacteria, such that the river sample was composed of HighDNA bacteria up to 90% of total bacterial abundance whereas LowDNA bacteria dominated in the lacustrine part of the reservoir (%HDNA ~ 30). Average bacterial fluorescence, which is related to average bacterial size (Gasol and del Giorgio 2000), varied strongly from side to side of the reservoir (Fig. 2B). Bacteria in the riverine part of the reservoir had also higher growth rates and higher total Leu and TdR incorporation rates (Fig. 2C) than that on the lacustrine side. Although our Leu data seem to underestimate growth rates, Leu uptake paralleled TdR uptake (Pearson’s R = 0.73, n = 10, P = 0.016) and thus became a good estimator of bacterial activity and growth. We estimated bacteria to have growth rates above 1 d⁻¹ in the river and ~0.1 d⁻¹ in the Dam. Again, conspicuous changes in growth rates occurred at 8–12 km from the Dam, partly coinciding with the river
The transplant experiment was done with water from 1.2 km from the Dam ("Dam" water) and water from 14.2 km from the Dam ("River" water). These two samples were different in physicochemical characteristics (Table 1): Eh, pH, oxygen, nutrients, etc., but were also different in a series of other parameters. Flagellates and ciliates were much more concentrated in the River sample than in the Dam sample: \(5 \times 10^3\) versus \(2 \times 10^3\) flagellates \(\text{ml}^{-1}\) and 94 versus 7 ciliates \(\text{ml}^{-1}\), respectively. Thus, the estimated grazing impact was five times higher in the River than in the Dam (Table 1), which imposes a much higher loss rate to bacteria. Correspondingly, bacteria were larger, had more DNA, incorporated more total and per cell TdR and leucine, and were growing at a higher rate in the River than in the Dam. In fact, even though total abundance was similar in the River and in the Dam, there were four times fewer LowDNA bacteria and twice the number of HighDNA bacteria in the River than in the Dam (Table 1). In terms of rough community composition, the River community was very different from the Dam community: 79% versus 30% %HDNA (Table 1), more FISH detectability in the River (59% vs. 43%), and higher abundance of CF bacteria in the River with more EUB+ not belonging to any of the used group-specific probes in the Dam (Fig. 3). The DGGE (Fig. 3) also showed strong differences: the number of bands was different (12 in the Dam and 17 in the River), the most conspicuous bands in the River corresponded to a CF-like organism and to an algal chloroplast (Chl \(a\) was 36 mg m\(^{-1}\)), and the most conspicuous bands in the Dam corresponded to a cyanobacteria-and a Gram-positive-like organism.

According to the data presented, we would expect bacteria in the River not to be nutrient-limited and be strongly determined by the large (Table 1) predatory impact. But would bacterial abundance, biomass, activity, and growth rate be all regulated by predators? How would community composition change in response to the lack of predation? We intended to answer these and related questions with the dialysis bag transplant experiment.

As a general rule, bacteria grew in almost all treatments. The Dam sample bacteria grew more in the transplanted treatments (Fig. 4, Table 2). The filtered (BF) and CTL samples evolved in parallel, but bacteria in the BF treatment grew less than those in the TR + BF sample. The %HDNA bacteria increased significantly (Table 2) in both TR samples and slowly in the samples incubated in the Dam. The River sample responded differently from the Dam sample: maximal growth was recorded in the BF treatment. The TR BF treatment also grew above the CTL. However, in the unfiltered but transplanted treatment, bacteria grew little and the %HDNA decreased with time.

A similar pattern could be observed in the cell-specific characteristics (Fig. 5, Table 2). The Dam sample bacteria increased apparent size (light scatter) and DNA content (Syto13 fluorescence, Fig. 5A) in the TR treatments. Cell-specific Leu uptake also increased in the TR treatments but slightly less in the TR + BF treatment. It is interesting to
Table 2. Treatments that induced the maximal concentrations or rates of growth for each of the sampled waters and significance of their differences. The treatments are ordered such that the one that conferred maximum values is placed in the first position, then the second one, and so on. The significance tests are from Bonferroni-corrected post hoc tests or t tests (see text for details).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Dam water</th>
<th>River water</th>
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<tbody>
<tr>
<td>Low DNA bacteria abundance</td>
<td>TR + BF ≫ TR = BF = CTL</td>
<td>BF &gt; CTL = TR = TR + BF</td>
</tr>
<tr>
<td>High DNA bacteria abundance</td>
<td>TR + BF ≫ TR &gt; BF = CTL</td>
<td>BF &gt; TR + BF = CTL = TR</td>
</tr>
<tr>
<td>All bacteria abundance</td>
<td>TR + BF &gt; TR ≫ BF = CTL</td>
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<td>% HDNA</td>
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<td>SSC</td>
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<td>CTL ≫ BF = TR + BF = TR</td>
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<td>Green fluorescence</td>
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<td>CTL = TR + BF ≫ BF &gt; TR</td>
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<td>CTL ≫ TR</td>
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<td>Cil abundance</td>
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<td>CTL &gt; TR</td>
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<td>EUB+ abundance</td>
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<td>ALF abundance</td>
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<td>TR + BF = BF &gt; CTL = TR</td>
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<td>BF = CTL = TR = TR + BF</td>
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<td>BF ≫ CTL = TR = TR + BF</td>
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<td>All bacteria SGR</td>
<td>TR + BF ≫ BF &gt; TR = CTL</td>
<td>BF ≫ CTL = TR = TR + BF</td>
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>, P between 0.05 and 0.005; ≫, P < 0.005. CTL, control; BF, filtered, bacterivore-free; TR, transplanted; TR + BF, filtered and transplanted; SSC, 90° light scatter; LIR, leucine incorporation rates; CS-LIR, cell-specific leucine incorporation rates; SGR, specific growth rate; ALF, α-proteobacteria; BET, β-proteobacteria; GAM, γ-proteobacteria; CIL, cytophaga/flavobacterium bacteria.

note that it took some time for the samples to adapt to the new conditions, and the 12 h samples were very different from the 24 h samples. Again, the River sample responded differently: in all treatments, bacteria decreased their apparent size and DNA content in the first 12 h, less so in the CTL. Cell-specific activity (SGR, Fig. 5C) also decreased in all treatments in the first 12 h, but in the CTL and the TR experiment less than in the BF samples. In that case, reduction of the predation pressure decreased the specific activity of the bacteria.

Total bacterial activity was only measured with Leu in the experiment, and it was strongly dependent on the nutrient availability (Fig. 5B, Table 2). Thus, the Dam sample significantly (Table 2) had much higher activity when transplanted. Filtration had no effect at all. It is interesting to see that bacterial activity did not start to increase until time 12 h. The combination of bacterial activity and biomass, specific growth rate, also increased in the TR treatments but slightly less in the TR + BF. In the River sample, the nutrient environment also regulated total bacterial activity, given that the maximal activity was recorded in the nontransplanted samples. However, the BF sample had higher total activity. Similarly, more activity could be measured in the TR + BF treatment than in the TR-only treatment. Growth rate (Fig. 5C) decreased in all treatments, but in the CTL and the TR experiment less so than in the BF samples. Reduction of the predation pressure decreased here bacterial growth rate.

Ciliates and flagellates increased mainly in the samples incubated in the River: the Dam sample stayed at 5,000 HNF ml⁻¹ when incubated in the Dam but increased to 7,000 HNF ml⁻¹ when incubated in the river. Similarly, the Dam sample stayed at ~5,000 HNF ml⁻¹ when incubated in the Dam but increased to 9,000 HNF ml⁻¹ when incubated in the river. A similar trend was apparent in the ciliate abundance data and, thus, in the bacterial total losses due to grazing. Nutrient availability directly affected protozoan abundance and the bacterial losses to grazers.

The rates of growth of the different bacterial subgroups determined with the group-specific oligonucleotide probes were different (Fig. 6) and contributed to changes in the final composition of each group to community composition (Table 3). The effects of the different treatments on the rates of growth of each subgroup were different than on the growth of the bulk bacteria (shown at the right of both graphs in Fig. 6). Except for a few of the subgroups, the rates of change between times 0 and 23 were strongly correlated to the rates at 0–47 h (n = 61, r = 0.90, P < 0.00001), and that allowed us to show only the rates of growth between 0 and 47 h in Fig. 6.
In the Dam sample, the TR + BF treatment was the one that elicited the maximal growth in all of the groups (Fig. 6A). In that treatment, ALF-, BET-, GAM-proteobacteria and CF bacteria grew at approximately the same rates, whereas in all other treatments GAM-proteobacteria grew at higher rates than the other subgroups. ALF-proteobacteria grew at a lower rate in all treatments. However, the rates of growth of ALF-proteobacteria in the TR + BF treatment were as large as those of the other subgroups in the other treatments. Because the response of the different subgroups to the treatments were so similar, final BCC was also relatively similar in the different treatments (Table 3), except for the TR + BF treatment, which had a markedly higher contribution of CF bacteria (25% vs. 12%–15%) and ALF-proteobacteria (3% vs. 0.4%–1.8%).

In the River sample, the treatments affected very differently each bacterial subgroup (Fig. 6B). ALF- and GAM-proteobacteria grew faster in the samples incubated in the same river (CTL and BF) and less in the samples incubated at the Dam site. BET-proteobacteria, however, grew faster in those samples that were not filtered (CTL and TR) and less in those samples that were filtered. CF bacteria grew faster in the BF sample and much more slowly in all other treatments. GAM-proteobacteria were the faster growers in all the treatments, and CF bacteria were less stimulated by the incubation. Altogether, these differential stimulation effects on the different subgroups contributing to BCC translated into BET-proteobacteria dominating in the unfiltered samples and CF in the filtered samples (Table 3). ALF-proteobacteria were more relevant members of the community also in the filtered (8%) than in the unfiltered (3%–5%) treatments, and GAM-proteobacteria were more important in the samples incubated in the river (9%–11%) than in those incubated at the Dam (4%–7%). Both ALF- and GAM-proteobacteria increased their contribution to BCC in all treatments, BET-proteobacteria did so only in the unfiltered samples, and CF decreased their relevance in the unfiltered treatments (Table 3).

Discussion

We performed a “transplant” experiment designed to test several hypotheses about the controls of bacterial abundance, activity, growth rate, and physiological and phylogenetic community composition in two contrasting sites in a eutrophic environment. Prior to discussing our results, some of the methods we used deserve consideration. We encountered very different values of bacterial production when measured with TdR or with Leu, and the ratio of both uptake rates (Leu : TdR) was 0.5–2 when it commonly varies between 5 and 40 (Chin-Leo and Kirchman 1990). Also, the estimates of bacterial production based on Leu uptake did not fit the measured net values in the bags (data not shown). Schuster et al. (1998) provide an explanation for our reduced values of Leu uptake rates in this system in arguing that colloidal organic matter can adsorb labile organic molecules (such as Leu), so that the rate of use of the labile molecules is re-
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Table 3. Community composition at the beginning and end of the incubations. Percentages over total DAPI counts.

<table>
<thead>
<tr>
<th>Dam sample</th>
<th>% EUB+</th>
<th>% ALF</th>
<th>% BET</th>
<th>% GAM</th>
<th>% CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>43.4</td>
<td>2.6</td>
<td>10.5</td>
<td>0.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Control, CTL</td>
<td>56.3</td>
<td>1.8</td>
<td>22.3</td>
<td>4.3</td>
<td>15.5</td>
</tr>
<tr>
<td>Transplanted, TR</td>
<td>56.4</td>
<td>0.4</td>
<td>15.1</td>
<td>5.7</td>
<td>12.2</td>
</tr>
<tr>
<td>Filtered, BF</td>
<td>59.0</td>
<td>0.4</td>
<td>11.7</td>
<td>4.9</td>
<td>13.6</td>
</tr>
<tr>
<td>Filtered and transplanted, TR + BF</td>
<td>59.4</td>
<td>3.1</td>
<td>25.6</td>
<td>4.7</td>
<td>24.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>River sample</th>
<th>% EUB+</th>
<th>% ALF</th>
<th>% BET</th>
<th>% GAM</th>
<th>% CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>59.2</td>
<td>1.8</td>
<td>10.3</td>
<td>1.0</td>
<td>33.6</td>
</tr>
<tr>
<td>Control, CTL</td>
<td>64.3</td>
<td>5.5</td>
<td>40.4</td>
<td>9.3</td>
<td>18.5</td>
</tr>
<tr>
<td>Transplanted, TR</td>
<td>76.0</td>
<td>2.6</td>
<td>30.2</td>
<td>3.8</td>
<td>21.4</td>
</tr>
<tr>
<td>Filtered, BF</td>
<td>70.1</td>
<td>7.8</td>
<td>8.6</td>
<td>10.5</td>
<td>30.4</td>
</tr>
<tr>
<td>Filtered and transplanted, TR + BF</td>
<td>65.6</td>
<td>8.0</td>
<td>13.3</td>
<td>6.9</td>
<td>30.8</td>
</tr>
</tbody>
</table>

Reduced (by as much as two orders of magnitude), Kirschner and Velimirov (1999) have also shown that in the presence of large colloidal dissolved organic matter (DOM), the efficiency of protein extraction may be low unless protein co-precipitants are added. Large amounts of colloidal DOM (as found on marine snow, in organic flocs, and in Sau) could thus underestimate the true values of bacterial production. As much as 50% of bacteria in Sau can be more or less loosely attached to these flocs, and the dominance of bacteria belonging to the CF cluster in the reservoir has probably to do with that fact (Šimek et al. 2001), given the commonly reported association of CF bacteria with aggregates (Weiss et al. 1996). However, and given that we found a similar trend in Leu and TdR uptake along the reservoir (Fig. 2) with a good correlation, we consider that our values of Leu uptake in the bags are reasonable estimates of bacterial activity, at least comparable between treatments.

We used the %HDNA bacteria, as detected in flow cytometric analysis of Syto13-stained bacteria, as an index of the proportion of “active” bacteria in the community (Gasol et al. 1999). The %HDNA can be taken as an index of physiological community structure: using cell sorting, Servais et al. (1999) have shown that Leu incorporation and growth rate was higher in the bacteria that had more DNA-induced Syto green fluorescence. Further research has shown that most (>80%) Leu uptake is associated to the HighDNA bacteria (Lebaron et al. 2001). None of the methods currently in use for splitting the bacterial community into their active and inactive component is completely satisfactory, because each of the methods measures a different cell property: cell membrane integrity, detectable respiratory activity, DNA in a compacted way, or enough RNA as to have protein production activity (see discussion in Gasol et al. 1999). In all instances, however, the presence of a positive fluorescent signal after hybridization with an oligonucleotide probe has been considered to be a signal of cellular activity (Poulsen et al. 1993). In the Sau reservoir, we found, in general, that the large bacterial cells in the river inflow also had the maximal Syto fluorescence (which is induced by DNA in oligotrophic samples but could also be induced by RNA in more eutrophic sites) and the strongest FISH fluorescence signal, which indicates a large RNA content per cell. We also found a very good relationship between the concentrations of HighDNA bacteria and the concentrations of cells that hybridized with the eubacteria general probe used (EUB-detectable, Fig. 7B). The relationship between all bacteria and EUB-detectable bacteria was good but had a slope significantly smaller than 1 (Fig. 7A), whereas LowDNA bacteria and EUB-detectable bacteria were, as expected, not correlated (Fig. 7C). There was a relatively good correspondence between the concentrations of EUB-undetectable bacteria and the LowDNA bacteria (Fig. 7D), but, as has also been encountered elsewhere (Gasol et al. 1999), the correlation was not perfect and forced the values of %HDNA and %EUB+ to be in the same ballpark but not well correlated (Fig. 7E). The good relationship that we present between the amount of HighDNA bacteria and the EUB-detectable cell abundance reinforces our hypothesis that the cells with higher DNA content are the most active ones in the community, are those that respond immediately to changes in predation pressure and nutrient availability, and the differentiation of these bacteria after flow cytometric analysis of Syto13 is the simplest and fastest way of separating the active members of a bacterial community (i.e., Gasol et al. 1999).

**Controls of abundance and production**—We used the concept of a “transplant” experiment, in which organisms are allowed to grow in an environment different from the one they were growing: dialysis bags that acted as a sort of “cage,” providing bacteria with access to a changed environment. We further used filtration to remove grazing pressure in some treatments and thus manipulate the factors that were expected to regulate the different characteristics of bacterial communities. Dialysis bags are not a perfect cage, but they are much more realistic than any experiment in bottles.

Our strategy clearly served the original purpose, because we were able to identify a few consistent patterns (Table 2). Predation was the main factor regulating bacterial abundances in the river. Bacterial production and the cell-specific measures of growth were higher in the CTL treatments but showed evidence of a double control: by nutrient supply and by predators (Fig. 5). Thus, the BF sample produced even more than the CTL sample, and cell-specific Leu uptake was lower in the BF treatments. Our data support the hypothesis that the nutrient supply at the River was enough to allow for many more bacteria to exist, but predators did not allow that to occur. When released from predation, bacteria increased abundances linearly, which reflects density-depen-
Bacteria in a eutrophic reservoir

Fig. 7. Relationships between the abundances of (A) all bacteria, (B) HighDNA bacteria, and (C) LowDNA bacteria and the abundances of EUB-detectable bacteria. Also shown, (D) the relationship of EUB-undetectable bacteria and LowDNA and (E) the relationship between the %HDNA and the %EUB-detectable cells. The 1:1 line is always shown, as well as the regression lines when significant.
transplanted replicates only. We believe that the Dam community was closer to Wright’s type (3) and only after being transplanted to the river started to activate. These interpretations would be consistent with the differences in %HDNA bacteria at both sites: 29% in the lacustrine site and 78% in the riverine site (Table 1).

It is commonly assumed that the rate of bacterial production is controlled by the rate of supply of nutrients and that final abundances and the rates of specific growth are either determined both by predation pressure and substrate supply (Wright and Coffin 1984) or just by predators (Thingstad and Lignell 1997). Some analyses have suggested that predators alone will be more relevant in eutrophic sites and substrate supply in oligotrophic environments (Sanders et al. 1992). However, in compiling the results of several experimental and empirical studies, Pace and Cole (1994) found little evidence of systems where predators were limiting bacterial abundances. In whole-lake manipulation experiments, where fish manipulation cascaded down to different zooplankton community structures, these authors concluded that resource supply determined growth rates, predators limited bacterial growth, and their interaction determined bacterial biomass and production (Pace and Cole 1996). In that framework, our results are relevant in several ways: large amounts of allochthonous carbon and large chlorophyll concentrations would suggest that plenty of nutrients are available for bacterial growth in the river, but bacterial standing stock and, hence, production were limited by grazing (Table 3). Šimek et al. (2000) studied the rates of grazing of flagellates and ciliates on bacteria in reservoirs of different trophic levels. A general pattern of increasing ciliate importance with increasing system trophic status was detected and is also apparent in our samples: although HNF potential grazing was two–three times higher in the River side of the reservoir than in the Dam area (Table 1), ciliate potential grazing was an order of magnitude higher in the river, which stresses the importance of these organisms in that more nutrient-rich region of the reservoir. Small omnivorous, fast-growing ciliates of the Halteria type dominated the reservoir and seem to be key pico- and nanoplankton grazers in meso- to eutrophic environments (Šimek et al. 2000; Comerma et al. 2001). In the Sau reservoir, protistan grazing impact was much larger in the river, and this was reflected in the response of bacteria to the manipulations: river bacteria increased in the bacterivore-free samples (Table 2).

Although predators determined bacterial abundance in that river sample, this was not the case for the Dam sample (Fig. 4), because filtration alone did not elicit any growth and it was necessary to also increase bottom-up supply to generate a filtration effect. Production and activity were indeed regulated by nutrient supply and greatly increased in the samples incubated in the river (Fig. 5). However, filtration, independently of the incubation site, reduced the growth rate of the river water sample. It also modulated the effect of nutrient supply on bacterial activity (Fig. 5B), again for the river sample.

It is also interesting to remark that most of the relevant changes occurred during the first 24 h of the experiment. Most variables did not change between 24 and 48 h (see, e.g., Fig. 5). Whether the growth on the dialysis bag walls could have prevented more interchange with the outside after 24 h cannot be answered, but we tend to believe that the changes did not continue simply because a new transient steady state had been obtained after the initial perturbation. This type of experiments cannot reflect the long-term response of the bacteria to varying nutrient and predation fields and simply reflects the results of a shifting of these conditions. In answering one of our initial questions, changes in bacterial abundance, production, and activity were obvious at time frames of 24 h. Changes in BCC were not that clear in the case of the Dam sample but seemed to be large enough to be above detection limits in the River sample (Table 2).

Our data would then suggest that no rules are general for the factors that regulate bacterial characteristics even in a single freshwater environment. The results of the mathematical models from which some of the previous expectations were derived could be biased because of not taking into account the physiological and phylogenetic structure of the bacterial communities. In this experiment, although all bacterial subgroups (ALF-, BET-, and GAM-proteobacteria and CF bacteria) had higher rates of growth in the TR + BF treatment for the Dam sample, differences occurred in different groups of the River sample (Fig. 6 and Table 2). We discuss these data in the following section.

Controls on community structure.—In terms of physiological community structure, the Sau reservoir had very contrasting ends: although in the river side most of the bacteria had high DNA contents (Fig. 2), this value decreased toward the lacustrine part of the reservoir, changing from ~70%–90% %HDNA to ~30%. Parallelizing the measures of cell-specific activity, transplanting the Dam water to the river end produced an increase in %HDNA from 20%–30% to 60%–70% during the experiment (Fig. 4). The presence of predators only slightly diminished the final %HDNA. For the River sample, however, predators did seem to decrease the final %HDNA, even though few changes were evident in that value throughout the experiment (Fig. 4D). As was suggested above, the %HDNA seems to be well correlated to measures of system trophic and can thus be used as a surrogate of cell-specific activity.

In terms of phylogenetic BCC, and as discussed elsewhere (Šimek et al. 2001), the Sau reservoir is typical of a freshwater planktonic system in the significant role of BET-proteobacteria (i.e., Methé et al. 1998; Glöckner et al. 1999). However, and different from what the latter authors had found, CF bacteria mostly numerically dominate in Sau reservoir (see also Šimek et al. 2001). CF bacteria are frequently dominant in aggregates or flocs (Weiss et al. 1996), they tend to dominate particulate organic carbon colonization (Riemann et al. 2000), have been shown to dominate the uptake of high-molecular-weight DOM (Cottrell and Kirchman 2000), and are commonly relevant in rivers (Kenzaka et al. 1998) and anoxic lake depths (Casamayor et al. 2000). In a detailed image analysis study of BCC in Sau reservoir, Šimek et al. (2001) showed how the riverine BETA bacteria were mostly filamentous, whereas the CF were mostly medium-sized short rods. Because the CF bacteria dominate the riverine part of the reservoir but are less relevant in the la-
attraction (Fig. 5), community physiological structure (Fig. 4), physiological activities (del Giorgio et al. 1996). We show that there could be also an effect of substrate availability on BCC. A few reports of changes in BCC appeared (Fig. 6, Table 4) but that the final bacterial composition stays relatively stable (Table 3), as it occurred for three of the four treatments of the Dam water sample. Maximal rates of growth for GAM-proteobacteria in our bags were in contrast to the experiment reported by Jürgens et al. (1999), in which they did not develop at those high speeds (see Table 4). All rates were higher in Sau reservoir than in the system studied by these authors, which probably reflects the higher trophic level of the reservoir (Chl a in the fishless pond of Jürgens et al.’s study was <0.5 μg L⁻¹). Šimek et al. (1999) found small changes in BCC in some of their filtration treatments and claimed that it was related to small changes in protistan predation pressure. The most significant changes in BCC were primarily associated with the treatments that involved strong changes in protistan predation pressure (relative to preincubation conditions. However, our data suggest that it is possible that large rates of growth appear (Fig. 6, Table 4) but that the final bacterial community composition stays relatively stable (Table 3), as it occurred for three of the four treatments of the Dam water sample. Maximal rates of growth for GAM- and small growth (or even losses) for ALF-proteobacteria ended up being irrelevant, given that these groups had a very low share of bacterial abundance. In the fourth treatment, however, a relatively large rate of growth of ALF- and BET-proteobacteria sufficed to change BCC in the bacterivore-free and transplanted treatment (Table 3, Fig. 6). Inspection of Table 4, with the observation that all bacterial subgroups grew faster on average in the river samples than in the Dam samples, gives a small indication that at least some of the CF bacteria in the river sample were not actively growing in situ or supported allochthonously had very large rates of growth, large bacterial activities, and were predated at a large rate (Table 1). They were everything but inactive.

Both laboratory (Šimek et al. 1999; 2001) studies have demonstrated the power of protistan bacterivory to alter BCC, bacterial size structure (e.g., Jürgens and Güde 1994) and bacterial-specific metabolic and physiological activities (del Giorgio et al. 1996). We show here again this to be the case both in terms of cell-specific activity (Fig. 5), community physiological structure (Fig. 4), and phylogenetic composition (Table 3).

In a previous study elsewhere, Šimek et al. (1999) manipulated only predation pressure in Rimov reservoir and anticipated that there could be also an effect of substrate availability on BCC. Specifically relating to the BCC of the longitudinal transect in Šimek et al. (2001) suggested that the riverborne, large, and fast-growing bacteria adapted to large substrate concentrations would become less competitive and increase its mortality rate when they would encounter an area with higher predation pressure—particularly directed to the larger cells (the middle part of the reservoir) and less total amount of substrate supply or lower quality of this supply. In contrast, the lacustrine bacteria, with smaller-sized cells, could be better adapted to the largely autochthonous production, and win competitively. Part of the interest of the present study was in describing how both types of control compare in their effect on BCC.

Table 4 reports the average and maximal specific growth rates recorded for each bacterial subgroup in both experiments performed. Both BET- and GAM-proteobacteria grew at a high rate in the Dam samples that had been transplanted and were bacterivore-free. The GAM-proteobacteria also grew very fast in the River sample (see also Fig. 6). The GAM-subgroup of the proteobacteria has many phylotypes that are prone to growth when samples are enclosed, and dominate the culturable part of the community (Glöckner et al. 1999). The growth rates of GAM-proteobacteria in our bags were in contrast to the experiment reported by Jürgens et al. (1999), in which they did not develop at those high speeds (see Table 4). All rates were higher in Sau reservoir than in the system studied by these authors, which probably reflects the higher trophic level of the reservoir (Chl a in the fishless pond of Jürgens et al.’s study was <0.5 μg L⁻¹). Šimek et al. (1999) found small changes in BCC in some of their filtration treatments and claimed that it was related to small changes in protistan predation pressure. The most significant changes in BCC were primarily associated with the treatments that involved strong changes in protistan predation pressure, relative to preincubation conditions. However, our data suggest that it is possible that large rates of growth appear (Fig. 6, Table 4) but that the final bacterial community composition stays relatively stable (Table 3), as it occurred for three of the four treatments of the Dam water sample. Maximal rates of growth for GAM- and small growth (or even losses) for ALF-proteobacteria ended up being irrelevant, given that these groups had a very low share of bacterial abundance. In the fourth treatment, however, a relatively large rate of growth of ALF- and BET-proteobacteria sufficed to change BCC in the bacterivore-free and transplanted treatment (Table 3, Fig. 6). Inspection of Table 4, with the observation that all bacterial subgroups grew faster on average in the river samples than in the Dam samples, gives a small indication that at least some of the CF bacteria in the river sample were not actively growing in the initial natural community. Although we have some background information on the effects of predators on BCC, little is known in terms of the effects of variations in the nutrient supply on BCC. A few reports of changes in BCC after the collapse of algal blooms—i.e., increased nutrient supply but very rich in large polymeric DOM—support the

<table>
<thead>
<tr>
<th></th>
<th>Dam</th>
<th></th>
<th>River</th>
<th></th>
<th>Maximum in Jürgens et al. (1999)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Maximal</td>
<td>Treat</td>
<td>Time frame (h)</td>
<td>Average</td>
</tr>
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<td>ALF</td>
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<td>0.052</td>
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<td>0.055</td>
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<td>0.156</td>
<td>TR + BF</td>
<td>0–23</td>
<td>0.053</td>
</tr>
<tr>
<td>GAM</td>
<td>0.063</td>
<td>0.157</td>
<td>TR + BF</td>
<td>0–23</td>
<td>0.076</td>
</tr>
<tr>
<td>CF</td>
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<td>0.128</td>
<td>TR + BF</td>
<td>0–23</td>
<td>0.039</td>
</tr>
<tr>
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<td>0.079</td>
<td>TR + BF</td>
<td>0–23</td>
<td>0.045</td>
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<tr>
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<td>BF</td>
<td>0–11</td>
<td>0.037</td>
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<td>HighDNA Bacteria</td>
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<td>0.077</td>
<td>TR + BF</td>
<td>0–11</td>
<td>0.037</td>
</tr>
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<td>0.021</td>
<td>0.046</td>
<td>TR + BF</td>
<td>0–11</td>
<td>0.037</td>
</tr>
</tbody>
</table>
idea that CF are favored (van Hannen et al. 1999; Riemann et al. 2000), which is in accordance with the reported preference of these bacteria for high-molecular-weight polymers (Cottrell and Kirchman 2000). No such pattern was obvious in our Dam sample (Fig. 6), but an interesting pattern appeared in the River sample, given that ALF- and GAM-proteobacteria seemed to be much less favored by the transplant to the Dam than the other bacterial subgroups.

Our knowledge from previous experiments is that, after a sudden increase in predation pressure, only those species able to increase growth or those that generate some grazing-resistant strategy (Jürgens and Güde 1994) will survive. A sudden relief of grazing induces a strong shift in BCC caused by (1) an increasing proportion of the formerly selectively grazed strains or (2) the decreasing proportion of highly active strains that previously profited from the positive feedback of bacterivory (see Šimek et al. 1999). On the basis of previous experience, we would expect BET-proteobacteria to increase in systems without flagellate predatory pressure (Šimek et al. 1997) and ALF proteobacteria to increase when faced with strong flagellate predatory pressure (Jürgens et al. 1999; Šimek et al. 1999; Langenheder and Jürgens 2001). CF bacteria could also increase by forming flocs and predation-protected aggregates. In the Sau reservoir River sample, BET proteobacteria developed specially in the treatments which were not filtered, which would be contrary to expectations unless (1) filtration removed large predators—ciliates—and left small predators that increased predation on the BET proteobacteria (but our sample inspection did not support this explanation), or, most probably, (2) filtration reduced the initial abundance of the BET proteobacteria by removing the largest—and presumably faster growing—bacteria. Also in the River sample, CF bacteria grew only in the treatment that had been filtered, and this suggests that not only were CF bacteria growing in the river at a relatively low rate, but they were also impacted by grazers; thus, their contribution to BCC decreased in the unfiltered samples (Table 3). We would expect strong predatory pressure on these bacteria if, as reported by Šimek et al. (2001), the CF bacteria in Sau are small and morphologically edible. Finally, the data in Table 3 suggest that ALF proteobacteria increased slightly more in the filtered treatments than in those that had the intact microbial assemblage. The changes were too small to be significant, however.

We are aware of the extremely crude BCC characterization with these group-specific oligonucleotide probes. They are based on splitting the community into a few subgroups consisting of an unknown number of bacterial phylotypes. As an example of the problems associated to this approach, our proportions of EUB detectable cells were rather low (40%–70%) but were similar to those of most other freshwater studies (Alfreider et al. 1996; Glöckner et al. 1996; Weiss et al. 1996; Pernthaler et al. 1997; Glöckner et al. 1999; Jürgens et al. 1999; Šimek et al. 1999; Langenheder and Jürgens 2001). A more serious problem could be that a large number of EUB detectable bacteria remained unaffiliated with the very small set of probes that we used, and this could be related to the presence in these environments of organisms that would not be targeted by our probes (Gram-positive, as shown in Fig. 3). Even with these limitations in mind, the approach used in this study allowed us to identify the factors most likely to regulate bacterial abundance, activity, production, and community composition in this freshwater environment. It is obvious from the results presented that not only is the structure of the microbial communities variable in this longitudinally heterogeneous system (e.g., Šimek et al. 2001), but the factors that regulate the bacterial community vary in different parts of it.

References


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