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Influence of Top-Down and Bottom-Up Manipulations on the R-BT065 Subcluster of β-Proteobacteria, an Abundant Group in Bacterioplankton of a Freshwater Reservoir

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We studied the effects of nutrient availability and protistan grazing on bacterial dynamics and community composition (BCC) in different parts of the canyon-shaped Římov reservoir (Czech Republic). The effects of protistan grazing on BCC were examined using a size fractionation approach. Water from the dam area with only bacteria (<0.8 μm), bacteria and heterotrophic nanoflagellates (<5 μm), or whole water were incubated in situ inside dialysis bags. Top-down or predator manipulations (size fractionation) were also combined with bottom-up or resource manipulations, i.e., transplantation of samples to the middle and upper inflow parts of the reservoir with increased phosphorus availability. Significant genotypic shifts in BCC occurred with transplantation as indicated by denaturing gradient gel electrophoresis. Using different probes for fluorescence in situ hybridization, we found that 10 to 50% of total bacteria were members of the phylogenetically small cluster of β-proteobacteria (targeted with the probe R-BT065). These rod-shaped cells of very uniform size were vulnerable to predation but very fast growing and responded markedly to the different experimental manipulations. In all the grazer-free treatments, the members of the R-BT065 cluster showed the highest net growth rates of all studied bacterial groups. Moreover, their relative abundance was highly correlated with bacterial bulk parameters and proportions of bacteria with high nucleic acid (HNA) content. In contrast, increasing protistan bacterivory yielded lower proportions of R-BT065-positive and HNA bacteria substituted by increasing proportions of the class *Actinobacteria*, which profited from the enhanced protistan bacterivory.

Our knowledge of bacterioplankton community composition (BCC) in a broad variety of aquatic systems has significantly advanced with the use of various molecular techniques (see, e.g., references 14, 15, 16 and 51). However, much less is known about which factors regulate BCC in general and of individual bacterial groups in particular (4, 35, 37). Furthermore, the factors regulating BCC in highly dynamic aquatic systems or those with a large gradient of environmental conditions, such as river estuaries (3, 5, 7) or typical canyon-shaped reservoirs (11, 19, 26, 43), have received even less attention.

Canyon-shaped reservoirs are highly heterogeneous ecosystems. Often they have relatively short water retention times and display strong downstream longitudinal gradients in the factors potentially controlling bacterial dynamics and BCC. Thus, very distinct environmental conditions can be found in the same water body and at the same time. This heterogeneity has been explored to study how BCC is affected by various resource supply rates or grazing pressure encountered along such gradients (11, 43). Top-down manipulations have been seen to alter the balance between bacterial production and mortality in previous studies conducted in the Římov reservoir (44, 46), generating shifts in BCC (45). Distinct top-down manipulations generated different BCC (46), illustrating the specific role of both protistan selective grazing and various bacterial growth strategies (also cf. references 17, 21, and 31) in shaping overall BCC.

One general pattern that emerges from this previous work is that the impact of protistan grazing on BCC tends to be significant only under nutrient-limited conditions. Under such conditions, grazing pressure targeted to certain bacterial groups exceeds their growth rates, and changes in BCC result (43). In contrast, under resource-rich conditions (i.e., at the reservoir inflow), physiological shift-ups and accelerated bacterial growth are observed, and the changes induced by increased resource supply overwhelm losses due to grazing (43). However, some groups of bacteria have the potential for increases in abundance in spite of the strong grazing pressure, in particular those strains having a "fast growth and division strategy" (cf. reference 31).

The members of the R-BT065 cluster (a small group of the *Rhodoferax* sp. BAL47 cluster [51]; for the phylogenetic affiliation, see reference 46) can be an example of such bacterial

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opportunists. They can be abundant (up to 50% of total bacteria) and are found in many European and North American lakes (51, 52). The R-BT065 cluster contains sequences retrieved through cloning from lakes Grösse Fuchskuhle and Gossenköllesee (15), the Columbia River (6), and the Římov Reservoir, with a minimum similarity within the cluster of >96% (46). Little is known about the ecology and the trophic role of the members of this cluster in any environment. Our preliminary data indicate that they are very fast-growing rod-shaped cells of uniform size which are vulnerable to predation. While proportions of this group are stable or slightly increase with enhanced protistan bacterivory, they grow particularly well in bacterivore-free treatments (43, 46). The R-BT065 cluster must involve one or more bacterial phylotypes with a "fast growth and division strategy," thus representing true opportunistic strategists among the reservoir bacterioplankton.

In this study, we examined in detail the dynamics and growth capabilities of the members of the R-BT065 cluster as well as the overall shifts in BCC and activity structure under several different scenarios. Bacterioplankton were subjected to shifts in both bottom-up and top-down control in the canyon-shaped Římov reservoir. Shifts in BCC were monitored by using fluorescence in situ hybridization (FISH) with oligonucleotide probes and by denaturing gradient gel electrophoresis (DGGE). We estimated the proportions of bacteria with high nucleic acid (HNA) and low nucleic acid content cytometrically and rates of grazing-induced bacterial mortality. Our goals were (i) to assess short-term variability in BCC as a function of resource supply and predation rates, (ii) to determine the role of the R-BT065 cluster in changes in BCC and overall bacterioplankton dynamics; and (iii) to examine how the dynamics of the members of this cluster were related to changes in the proportions of HNA bacteria in the assemblage.

MATERIALS AND METHODS

Study site and experimental design. The experiment was conducted in the canyon-shaped, mesoeutrophic Římov Reservoir (South Bohemia [see reference 46 for details]). On 20 May 2002, 40-liter samples from the dam area (chlorophyll *a* concentration, 4.5 μg liter^{-1}) were collected from a depth of 0.5 m and mixed in a 50-liter plastic container. The experiment was conducted from 20 to 24 May at three experimental sites along the longitudinal profile of the reservoir (assigned as dam, middle, and river) during the clear-water phase of the lake. Water temperatures were 18 to 20°C at the dam and middle parts and 13 to 16°C at the river inflow. The experimental design combined top-down and bottom-up manipulations with plankton samples (see reference 43 for details). Briefly, duplicated, unfiltered <5-μm and <0.8-μm treatments were incubated in the dam site in dialysis bags with the latter two size fractions also incubated in parallel in bottles (see below). Also, <5-μm and <0.8-μm treatments originating from the dam site were transplanted into middle and river sites and incubated in dialysis bags. With this protocol, we ensured varied grazing pressures on the bacteria as well as nutrient supply rates.

We used a size fractionation approach to alter levels of bacterivory in natural populations (for details, see references 43 and 46) so that bacterioplankton was subjected to either negligible (<0.8 μm) or high (<5 μm) levels of protistan bacterivory. To assess the influence of resource availability, aliquots of size-fractionated plankton samples (see below) collected at the dam area were incubated in both dialysis bags (relatively free exchange of low-molecular-weight compounds) and bottles (no nutrient penetration). This was done at the DAM site characterized by moderate primary production (PP) of 16 μg of C liter^{-1} h^{-1} and a rather low concentration of dissolved reactive phosphorus (DRP) of 2 μg liter^{-1}. A parallel set of samples of the <0.8-μm and <5-μm fractions was transplanted and incubated in dialysis bags in the middle part of the reservoir (highest PP, 31 μg of C liter^{-1} h^{-1}; enhanced DRP concentration, 23 μg liter^{-1}), and finally, a third set of dialysis bags was incubated in the river inflow of the reservoir (moderate PP of 14 μg of C liter^{-1} h^{-1} but the highest concentration of DRP at 59 μg liter^{-1}).

The size fractions represented (i) "bacterivore-free" treatment via filtration through 0.8-μm-pore-size filters (47-mm diameter; OSMONIC Inc., Livermore, Calif.) which were assumed to remove all bacterivorous protists, (ii) "increased bacterivory" treatment via filtration through 5.0-μm-pore-size filters which removed the predators of heterotrophic nanoflagellates (HNF), and, in the DAM site also, (iii) control, untreated sample. For more details concerning filtration procedures, see the work of Šimek et al. (44, 46). The fractions incubated in dialysis bags are designated throughout the text as <5-μm, <0.8-μm, and "unfiltered" (UNF) treatments, respectively, and were completed with an identification of the site of the incubation, i.e., "dam," "middle," or "river." These water fractions were placed in ~2.5-liter pretreated (deionized water rinsed and boiled) dialysis tubes (diameter, 75 mm; molecular weight cutoff, 12,000 to 16,000 Da; Poly Labo, Switzerland). The samples incubated at the dam site in 2-liter glass bottles are also named with an identification of the type of the incubation, i.e., "bottle." All dialysis bag and bottle treatments were incubated at a depth of 0.5 m, oriented horizontally in open Plexiglas holders. Samples (~300 to 450 ml) were taken from each dialysis bag and bottle at 0, 24, 48, 72, and 96 h.

Bacterial abundance and biomass. Samples were fixed with formaldehyde (2% [vol/vol] final concentration), stained with DAPI (4′,6′-diamidino-2-phenylindole) (final concentration, 0.1 μg ml^{-1}, wt/vol), and enumerated by epifluorescence microscopy (Olympus BX 60). Cell volumes were measured using the LUCIA D (Lucia 3.52 [resolution, 750 by 520 pixels, 256 grey levels]; Laboratory Imaging, Prague, Czech Republic) semiautomatic image analysis system as previously described in detail by Posch et al. (32). Bacterial carbon biomass was calculated according to the method of Norland (29).

Bacterial production. Bacterial production was measured via thymidine incorporation modified from the method described by Riemann and Søndergaard (34) and described in detail by Šimek et al. (46). Site-specific empirical conversion factors between the thymidine incorporation rate and bacterial cell production rate were determined using data from the duplicate <0.8-μm treatments. The cell production rate was calculated from the slope of the increase of ln bacterial abundance over time (0, 24, and 48 h). Our determined ECFs, 0.84×10^{18}, 1.58×10^{18}, 1.36×10^{18}, and 1.16×10^{18} cells mol^{-1} of thymidine incorporated for bottle incubations and dam, middle, and river dialysis bag incubations, respectively, were used for calculations.

PCR and DGGE analysis. The bacterial 16S rRNA genes in the samples were amplified by filter PCR according to the protocol of Kirchman et al. (22). Sample fixation and filtration were performed as for preparing filters for FISH on polycarbonate filters. As templates, small pieces (~5 mm^2) of the 0.2-μm polycarbonate filters were used. The reactions (50-μl volume) contained 400 μM each deoxynucleoside triphosphate, 0.3 μM each primer, 3 mM $MgCl_2$, 1× PCR buffer, 1.25 units of *Taq* DNA Polymerase (QIAGEN), and 1.5 μl of bovine serum albumin (stock solution, 10 mg ml^{-1}; SERVA). We used the bacterial specific primer 358f (5′-CCT ACG GGA GGC AGC AG-3′), with a 40-bp GC clamp, and the universal primer 907r (5′-CCG TCA ATT CCT TTR AGT TT-3′), which amplifies a 550-bp DNA fragment of bacterial 16S rRNA (27). The PCR was performed with a Primus 96^{plus} thermal cycler (MWG Biotech) using the following program: initial denaturation step at 94°C for 5 min, 30 cycles of denaturation (at 94°C for 1 min), annealing (at 55°C for 1 min), and extension (at 72°C for 2.5 min), and a final extension step at 72°C for 5 min. PCR products were verified and quantified by agarose gel electrophoresis with a standard in the gel (Low DNA Mass ladder; GIBCO BRL). The products of three PCRs were pooled for each sample.

DGGE was carried out with a DCode Universal Mutation Detection system (Bio-Rad) as described previously by Muyzer et al. (27). We used 6% polyacrylamide gels with a gradient of DNA-denaturing agent from 20% to 80% (100% denaturing agent is 7 M urea and 40% deionized formamide). Comparable amounts of PCR product were loaded for each sample, and the gel was run at 100 V for 16 h at 60°C in 1× TAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with the nucleic acid stain SybrGold (Molecular Probes) for 45 min, rinsed with 1× TAE buffer, removed from the glass plate to a UV transparent gel scoop, and visualized in a UV transilluminator (LTF-Labortechnik, Wasserburg, Germany) with BioCapt software (LFT-Labortechnik). High-resolution images (768 by 568 pixels, 8-bit dynamic range) were saved as computer files (see reference 38 for details).

FISH with rRNA-targeted oligonucleotide probes. Analysis of planktonic BCC was carried out by in situ hybridization with group-specific Cy3-labeled oligonucleotide probes on membrane filters (1). To assess proportions of the *Actinobacteria* group (HGC69a probe), a modified catalyzed reporter deposition-FISH protocol was applied (see references 30 and 39 for details). Six different group-specific oligonucleotide probes (ThermoHybaid, Germany) were targeted to the

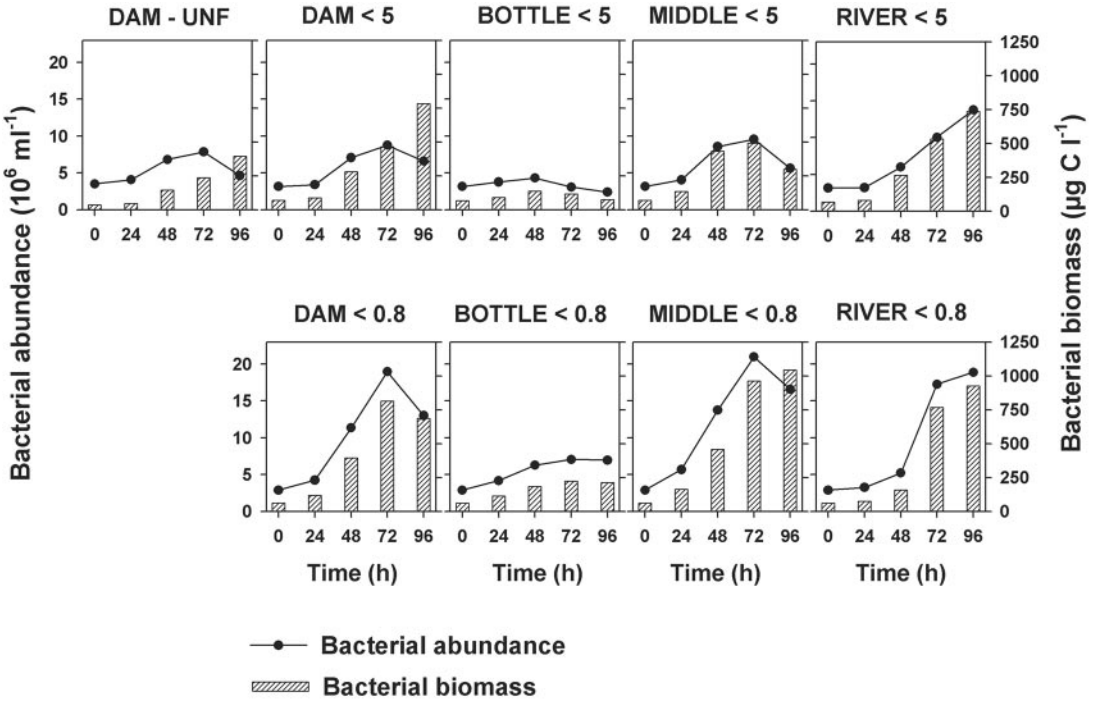

FIG. 1. Time course changes in bacterial abundance and biomass in different size fractionation treatments of <0.8 μm, <5 μm, and UNF reservoir water collected and exposed in dialysis bags at the dam area of the reservoir compared to the changes in the same <0.8-μm and <5-μm treatments but incubated in bottles or transplanted upstream to the middle and river inflow of the reservoir and incubated there in dialysis bags. Values are means for two replicate treatments. Error values are not shown to allow for a better visualization of the changes.

domain *Bacteria* (EUB338), to β and γ subclasses of the class *Proteobacteria* (the BET42a and GAM42a probes, respectively), to a narrower subcluster of the β-*proteobacteria* (R-BT065), to the *Cytophaga*/*Flavobacterium* group (CF319a), and to the *Actinobacteria* group (HGC69a). After the whole procedure, the filter sections were stained with DAPI and the proportions of hybridized bacterial cells were enumerated using an epifluorescent microscope (Olympus AX70 Provis).

Flow cytometric sample analysis. Subsamples were preserved with 1% paraformaldehyde plus 0.05% glutaraldehyde (final concentration), frozen in liquid nitrogen, and stored at −80°C. The samples were later thawed, stained for a few minutes with Syto13 (Molecular Probes) at 2.5 μM, and run in a desktop FACSCalibur flow cytometer (Becton Dickinson). We used MilliQ water as sheath fluid, samples were run at low speed (approximately 18 μl min^{-1}), yellow-green 0.92-μm Polysciences latex beads were used as internal standards, and the fixed samples were diluted two to four times with MilliQ water to keep the rate of particle passage below 500 particles per second to avoid coincidence (12). Bacteria were detected by their signature in a plot of side scatter versus green fluorescence, and we used the percentage of HNA (%HNA) bacteria as a simple way of describing the activity structure of the bacterial community (12, 13).

Protozoan grazing and enumeration. Protozoan grazing on bacteria was estimated using fluorescently labeled bacterioplankton (FLB) (42) concentrated from the reservoir water (see reference 46 for details). HNF and ciliate (only unfiltered treatment) FLB uptake rates were determined in short-term FLB direct-uptake experiments with FLBs equal to 10 to 15% of natural bacterial concentration. Subsamples for protozoan enumeration and tracer ingestion determinations (see reference 46 for details) were fixed with Lugol's formaldehyde decolorization technique (42). Five-milliliter (for flagellates) or 20-ml (for ciliates) subsamples were stained with DAPI, filtered through 1-μm black Poretics filters, and inspected via epifluorescence microscopy. To estimate total protozoan grazing, we multiplied the average uptake rates of ciliates and flagellates by their in situ abundances as previously described (46).

Chemical parameters. Dissolved organic carbon was analyzed on a Shimadzu TOC-5000A analyzer, and total and dissolved reactive phosphorus were determined spectrophotometrically according to a method described previously by Kopáèek and Hejzlar (23).

Statistical analysis. After arc-sine data transformation, two-way analysis of variance (ANOVA) was used to compare changes in the proportions of the respective genotypic groups of bacteria (as a percentage of total DAPI-stained bacteria) at 24, 48, 72, and 96 h induced by the top-down manipulation (i.e., size fractionation) and by the different bottom-up supply rates in the dam, middle, and river sites. Using duplicate data on cell number increase in <0.8-μm treatments, net doubling time and growth rate of different bacterial subgroups were calculated for intervals with exponential growth using nonlinear regression. We then tested (*F* test) for significant differences in growth rates of the bacteria targeted by the R-BT065 probe compared to those of total bacterioplankton and other phylogenetic groups of bacteria. All statistics analyses were performed using GraphPad Prism (GraphPad Software, San Diego, Calif.).

RESULTS

Bacterial and flagellate community dynamics and activity. In all treatments, bacterial numbers and biomass covaried closely but with remarkably lower values found in the bottle incubation (Fig. 1). In the grazer-free (<0.8-μm) dam, middle, and river dialysis bag treatments, bacterial numbers reached similar maxima and doubling times of 16 to 20 h (Table 1). However, the same treatment, incubated at the dam site in a nonpermeable bottle, showed much lower maximal abundance and longer bacterial doubling times of ~42 h.

Different trends were evident in the <5-μm treatments (Fig. 1). The increase in bacterial abundance observed during the first 48 to 72 h stopped, and then, with HNF exponential growth during the second half of the experiment (Fig. 2), bacterial abundances decreased markedly at 96 h. The only exception was the river <5-μm treatment, where bacterial numbers grew throughout the whole period, probably due to the inefficient grazing control by slow-growing HNF (cf. Fig. 1 and 2). The unfiltered treatment (dam UNF) showed fairly

TABLE 1. Net doubling times of ungrazed bacterioplankton and different bacterial subgroups[a]

Incubation site	Doubling time (h)						Interval (h)
	Bacterioplankton	R-BT065	BET42a	GAM42a	CF319a	HGC69a	
Dam	20**	8	13**	11	23*	23*	0–48
Bottle	42	19	29	60	32	35	0–48
Middle	20**	9	13	8	12*	18	0–48
River	16	9	11	10	14	12	24–72

[a] Shown are net doubling times of ungrazed bacterioplankton and different bacterial subgroups in the predator-free treatments (<8 μm) exposed at the dam area in dialysis bags (dam) and bottles compared to those transplanted and incubated in dialysis bags in the middle and river inflow parts of the reservoir. The values are means for two duplicate treatments. Rates were calculated for intervals with exponential growth using nonlinear fit of the duplicated data followed by testing significant differences in doubling times of the bacteria targeted by the R-BT065 probe compared to other bacterial groups. *, $P < 0.05$; **, $P < 0.01$.

similar trends in all microbial parameters as observed in the corresponding dam <5-μm treatment (Fig. 1 and 2), except for slightly lower values of bacterial biomass and production that might be related to the presence of metazooplankton grazing (20 cladocerans liter^{-1} [data not shown]).

Bacterial production (BP) in the grazer-free (<0.8-μm) treatments (Fig. 2) roughly reflected nutrient availability in the incubation site or treatment. Thus, in the bottle, BP was lowest and showed a decreasing trend. In contrast, much higher values and overall increasing trends were found in all dialysis bag incubations, with maxima of BP gradually increasing from the more P-limited dam site, through the middle site, and to the P-rich river site.

In all grazer-enhanced (<5-μm) and dam UNF treatments, changes in BP were strongly affected by the grazer population dynamics and bacterivory (Fig. 2). Thus, in most cases, BP increased only during the first 48 to 72 h and then markedly dropped due to grazing by the fast-growing HNF populations, peaking at 96 h (~10×10^3 to 59×10^3 individuals ml^{-1}). The only exception was the river <5-μm treatment, where HNF reached only ~4×10^3 individuals ml^{-1} and grazed <10% of BP at 96 h. This fact, together with the nutrient-rich conditions in the river site, yielded a continuous increase of BP throughout the study period. In contrast, in all other grazer-present treatments, total bacterivory approached (dam <5 μm), or exceeded, the level of BP at 96 h, sometimes severalfold (Fig. 2); see, e.g., these parameters in the bottle <5-μm treatment.

Changes in BCC. During the course of the study, the manipulations resulted in treatment- and site-specific changes in BCC as shown by both the fingerprinting (DGGE) and rRNA-

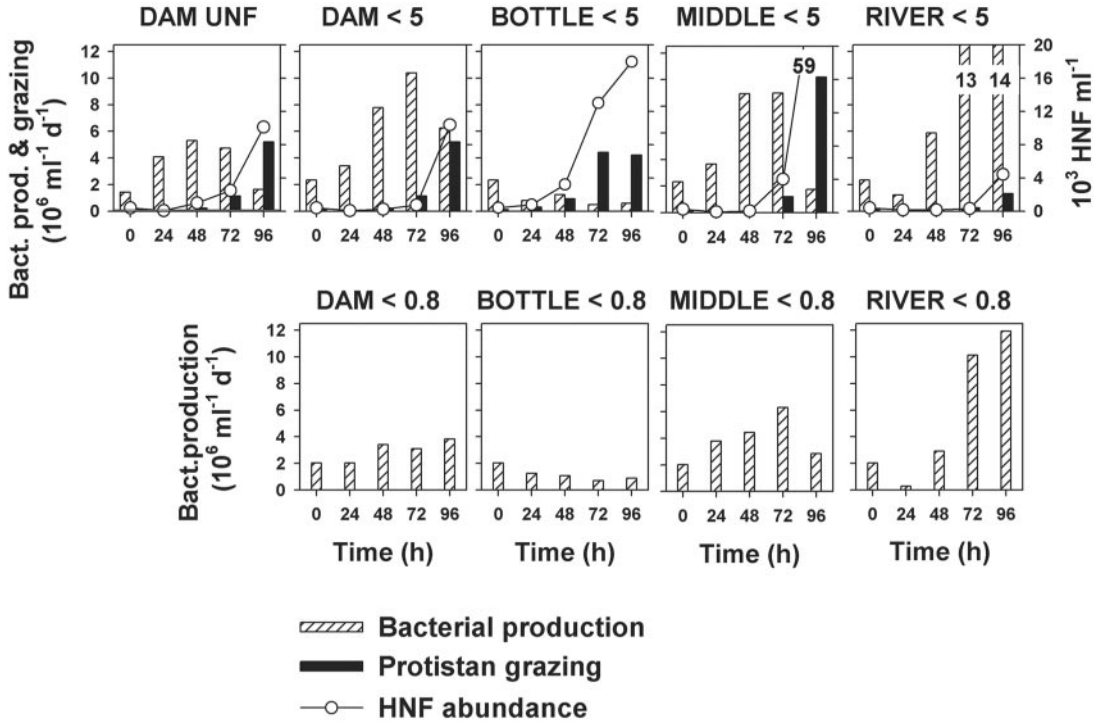

FIG. 2. Time course changes in bacterial production (Bact. prod.) and total protistan bacterivory in different size fractionation treatments of reservoir water collected at the dam area and incubated in bottles or in dialysis bags at the dam, middle, and river parts of the reservoir (for more details, see the Fig. 1 legend). Values are means for two replicate treatments. Note that "total protistan grazing" refers only to HNF bacterivory in all but the unfiltered treatment incubated at the dam site (DAM UNF), where ~20% of total grazing was caused by the ciliates present in the sample (data not shown).

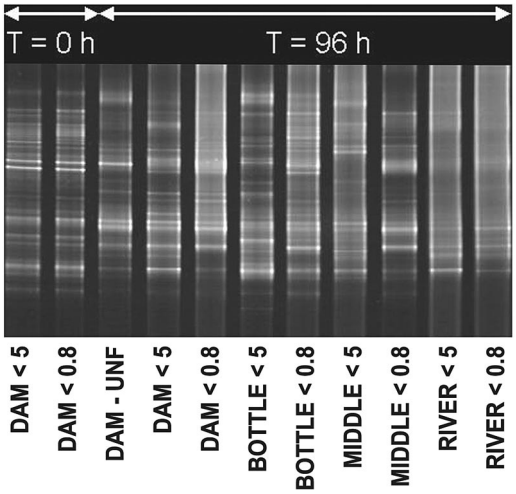

FIG. 3. Negative image of the bacterial 16S rRNA gene fragments separated by DGGE from the different size fractionation treatments of <0.8 μm and <5 μm at time zero compared to changes in banding patterns of samples incubated in bottles and in dialysis bags at the dam, middle, and river parts of the reservoir at the end of the incubation.

targeted oligonucleotide probes (Fig. 3 and 4). The initial DGGE banding pattern indicated that size fractionation itself had a negligible effect on BCC (Fig. 3). However, the incubation of the different size fractions and their transplantation upstream into areas differing in primary production and P availability induced marked shifts in BCC.

In the control samples at time zero, 19 DGGE bands were detected. At the end of the experiment, we found between 8 and 20 DGGE bands in each sample. The smallest band number was commonly found in both <0.8-μm and <5-μm river incubated treatments, suggesting a resource supply effect on diversity. Another general pattern was that the bacterivore-free treatments differed from the corresponding bacterivore-enhanced treatments, with the most marked differences detected within the bottle and middle treatments, where HNF exerted the strongest grazing pressure in the <5-μm treatments.

For the six different rRNA-targeted oligonucleotide probes we used in this study, cells detected by CF319a and HGC69a showed no clear trend in any treatment or sample group except for increases in proportions of HGC69a-positive cells in the bottle <5-μm treatment (Fig. 4). Commonly, cells detected with the general probe EUB338 and group-specific probes BET42a, R-BT065, and GAM42a showed initial increases reaching maximal proportions at 48 or 72 h, and then, they variably decreased towards the end. However, the proportion of cells detected with probe GAM42a also showed different trends: in the bottle <0.8-μm treatment, they slowly decreased, while in the river <0.8-μm treatment, they markedly increased during the second half of incubation.

A few additional common trends were also observed in all incubation sites. For instance, the proportion of HGC69a-detected cells (Fig. 4) was generally much higher in grazer-enhanced (<5 μm, 16% ± 5% [mean ± standard deviation]) than in the grazer-free (<0.8 μm, 8% ± 3%) treatments. In the latter treatments, cells detected with the specific R-BT065 probe for a small subcluster of β-proteobacteria mostly overgrew other β-proteobacteria (BET42a probe) within the first 48 h (e.g., middle and river in Fig. 4). Between 48 and 96 h of the experiment, we observed marked but not significantly higher proportions of the R-BT065 subcluster in the absence of grazing (34% ± 12%) compared to that in its presence (20% ± 9%). The fast growth of the R-BT065-positive cells also yielded the shortest net doubling time of all studied groups (Table 1) that was significantly ($P < 0.05$; F test) faster than that of other groups of bacteria at dam and middle sites, while these differences were statistically insignificant in river and bottle incubations. The R-BT065 subcluster displayed large growth potential and responded highly significantly (two-way ANOVA) to both types of manipulation, while cells detected with the probes CF319a and GAM42a responded either to transplantation or to different grazing levels, respectively (for details, see Table 2). The members of the class *Actinobacteria* (HGC96a probe) were significantly affected by the manipulation of the grazing pressure (Table 2, cf. Fig. 4).

Bacterial cytometric fingerprints. In general, the %HNA increased during the experiments, largely coinciding with increases in bacterial abundance and production (cf. Fig. 1, 2, and 5). In the grazer-free treatments, HNA cells were initially ~30% of total DAPI-stained bacteria (Fig. 5) and reached proportions of 50, 68, 75, and 80% in bottle, dam, middle and river, respectively, at 96 h. Interestingly, all grazer-present treatments showed initially similar trends as did bacterivore-free ones, but between 72 and 96 h, along with markedly increasing protistan bacterivory, HNA bacteria diminished in all but the river <5-μm treatment (Fig. 5), the one with relatively low HNF bacterivory (Fig. 2).

Statistical relationships. When data were pooled across all treatments, no significant relationship was found between the proportions of HNA bacteria and HNF abundance or bacterivory rates (Table 3). However, the %HNA bacteria was significantly and positively related to bacterial abundance, production, biomass, and mean cell volume (MCV). This indicated that the HNA bacteria are relatively large and likely the most active ones, shaping the overall dynamics of the bacterial assemblage. More specifically, we also detected that among the phylogenetic bacterial groups studied, changes in the proportions of cells targeted by BET42a and R-BT065 probes explained the largest part of the variability in %HNA bacteria (66 and 63%, respectively) (Fig. 6B and C). Moreover, when only bacterivore-free treatments were considered, >70% of the variability in %HNA bacteria was explained by changes in the proportions of the narrow R-BT065 cluster (Fig. 6C). Overall, all but one probe-defined bacterial group (*Actinobacteria*) significantly correlated with %HNA bacteria, and the importance of the relationship (as the r value in Table 3) roughly corresponded to the net growth rate achieved in the bacterivore free-treatments shown in Table 1.

The clear importance of β-proteobacteria, particularly of the R-BT065 subcluster, as one of the forces driving the dynamics of the reservoir bacterioplankton was also evidenced by the highly significant correlations of these probe-defined bacterial subpopulations and the basic bacterioplankton parameters shown in detail in Table 4.

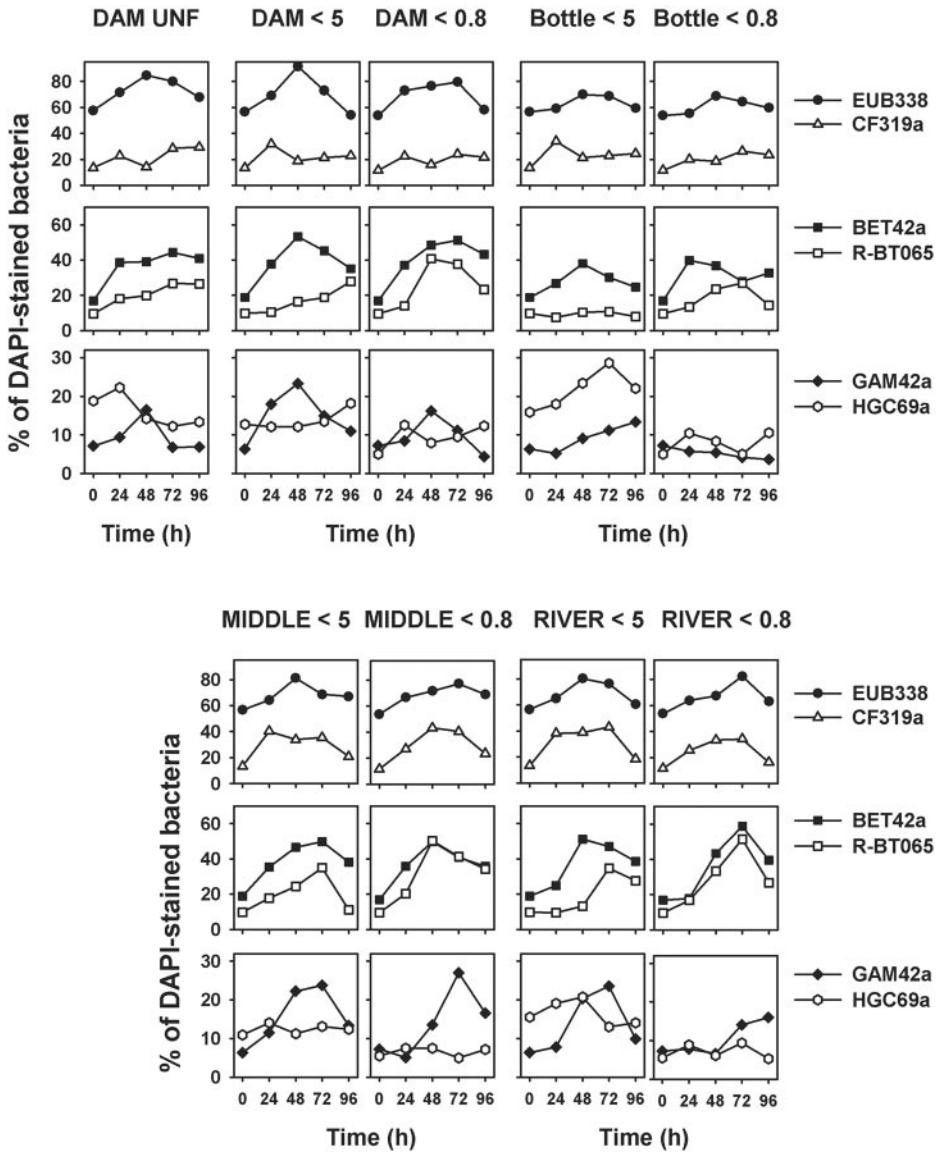

FIG. 4. Time course changes in the phylogenetic composition of the bacterioplankton community in different size fractionation treatments of reservoir water collected at the dam area and incubated in bottles or in dialysis bags at the dam, middle, and river parts of the reservoir (for more details, see the Fig. 1 legend). Shown are proportions of total FISH detection rates by the general bacterial probe EUB338, β-proteobacteria (BET42a), and the cells detected by the probe R-BT065, γ-proteobacteria (GAM42a), the *Cytophaga*/*Flavobacterium* group (CF319a), and the *Actinobacteria* group (HGC69a). Values are means of two replicate treatments.

DISCUSSION

Grazing has an effect on bacterial community composition. Our results show this through changes in the banding pattern in the DGGE (Fig. 3) and, as reported in previous studies, changes in the relative importance of defined groups as determined using FISH (cf. references 21, 44, and 46). However, by altering resource through transplantation, our data also demonstrate the effects of bottom-up factors in structuring BCC (Fig. 3).

We anticipated that the markedly different DRP concentrations at the three incubation sites would have a large influence on various bacterial parameters. By comparing the two most distinct sites in terms of DRP concentrations, i.e., dam and river, this assumption was only partially confirmed. For instance, bulk bacterioplankton production and growth rate and phylotype-specific growth rates of bacteria were highest in the river site, with no significant differences in growth rates compared to those of the fastest-growing cells, those detected with the R-BT065 probe (Table 1). In contrast, R-BT065-positive cells grew in dam <0.8-μm treatment significantly faster than other lineages, but the bulk bacterioplankton production and growth rate (Fig. 2 and Table 1) were only slightly lower than those of the river <0.8-μm treatment. Overall, these small differences did not fully reflect the large site-specific differences in DRP availability that were suggested in a previous experiment to have marked effects on the dynamics of the reservoir bacterioplankton and virioplankton (43, 49).

TABLE 2. Two-way ANOVA used to test for differences in BCC between the dam, middle, and river incubations throughout all experimental variants and different time intervals[a]

Probe	Bottom-up manipulation[b] (*F* value)				Top-down manipulation[c] (*F* value)			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
BET42a	3.16	0.35	1.49	0.18	0.22	0.72	0.65	1.13
R-BT065	**15.3****	**80.5*****	**6.12***	0.48	**29.4*****	**583*****	**18.8****	**6.24***
GAM42a	0.27	0.36	1.79	3.86	**6.15***	**11.3***	2.32	2.12
CF319a	0.66	**27.1****	**8.45***	0.96	3.07	0.09	0.02	0.01
HGC69a	1.09	2.94	0.61	2.28	**10.5***	**43.3*****	**6.4***	**6.2***

[a] We tested the differences in the relative contributions (as a percentage of total DAPI-stained bacteria) of five different phylogenetic groups of bacteria targeted by the probes BET42a, R-BT065, GAM42a, CF319a, and HGC69a induced by transplanting the samples from the dam area upstream to the middle and river areas and by manipulating top-down control via size fractionation of water samples. The significant differences are in boldface type. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[b] Dam samples transplanted to the middle and river parts.
[c] Effects of distinct grazing pressures when bacterivore-free treatments are compared to bacterivore-enhanced treatments.

Several different aspects might explain this apparent paradox between the detected DRP concentration and the growth rates achieved by bacterioplankton in different parts of the reservoir. (i) The pool of DRP can consist of very different ratios of bioavailable and nonbioavailable fractions (28, 36). (ii) Moreover, rapid turnover rates of the bioavailable P during the clear-water phase can override the effect of the analytically relatively low detectable DRP concentrations in the dam area (28), where nutrient recycling could be strongly stimulated by grazing activity of the abundant zooplankton (26). (iii) Although the FISH data with group-specific probes did not show large differences in BCC, the DGGE banding pattern at 96 h indicated remarkable site-specific BCC shifts. Thus, different bacterial phylotypes (cf. Fig. 3 and 4) were the major bacterial players at different parts of the reservoir, phylotypes which were well adapted to local ambient nutrient conditions allowing them to efficiently compensate for the variable P availability.

Bacteria in the bottle incubations showed dramatic differences in both overall dynamics and community composition compared to dialysis bag incubations. The bottle treatments always showed the lowest bacterial numbers, biomass, and growth rates of the different phylogenetic groups of bacteria (cf. Fig. 1 through 4). It indicates a specific "bottle effect" (10) and an overall problematic nature of bottle incubations for studying in situ microbial processes.

Additional differences were found between bottle and dialysis bag incubated populations. For instance, the treatment-specific BCC shifts (Fig. 3 and 4) supported different HNF populations. In bottle <5-μm incubations, very small and fast-growing *Spumella*-like chrysomonads dominated (see the rapid HNF increase in Fig. 2). Thus, the altered nutrient availability in bottle compared to dialysis incubations was associated with quite complex changes in the microbial food web: it affected not only BCC directly but also the grazer community composition and in turn also the HNF species-specific grazing, which probably differently impacted the resulting BCC as has been shown before (31, 33).

The pooling of data across all dialysis bag treatments showed distinct effects of the bottom-up and top-down manipulations on the relative contributions of different phylogenetic groups of bacteria (Table 2). Manipulation of the grazing pressure consistently altered the proportion of the *Actinobacteria* group

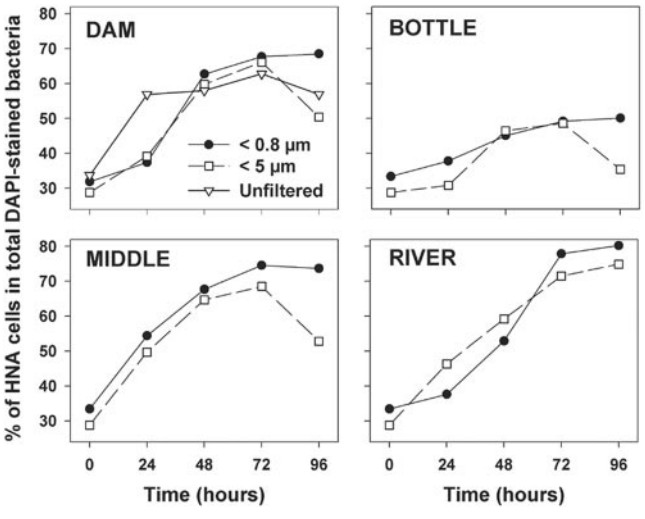

FIG. 5. Time course changes in the proportions of bacteria with HNA content related to total DAPI-stained bacteria in different size fractionation treatments of reservoir water collected at the dam area and incubated in bottles or in dialysis bags at the dam, middle, and river parts of the reservoir (for more details, see the Fig. 1 legend). Values are means for two replicate treatments.

TABLE 3. Correlation relationships between the proportion of HNA cells within the bacterial assemblages compared to HNF and bacterioplankton parameters and the proportions of FISH-detected subgroups of bacteria across all experimental treatments[a]

Parameter	Correlation coefficient (*r*)	Significance (*P*)
HNF abundance	0.045	0.8302
Total HNF grazing	0.063	0.7641
Bacterial abundance	0.846	<0.0001
Bacterial production	0.715	<0.0001
Bacterial biomass	0.845	<0.0001
Bacterial mean cell volume	0.813	<0.0001
% EUB338	0.643	<0.0001
% BET42a	0.814	<0.0001
% R-BT065	0.792	<0.0001
% GAM42a	0.585	<0.0001
% CF319a	0.461	0.0014
% HGC69a	−0.016	0.2912

[a] For calculations, the proportions of HNA bacteria and FISH-detected bacterial subgroups were expressed as a percentage of total DAPI stained cells. For all analyses, $n = 45$, except for those with HNF abundance and grazing ($n = 25$). Significant relationships ($P < 0.01$) are shown in boldface type.

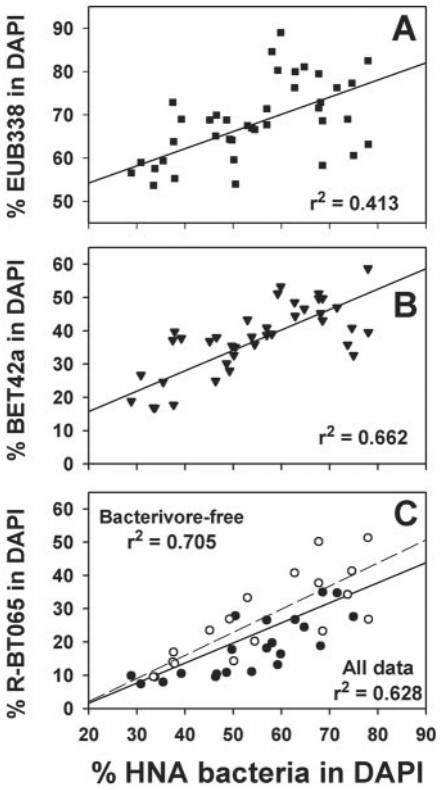

FIG. 6. Relationships between the proportions of bacteria with HNA content related to total DAPI-stained bacteria and proportions of probe-defined bacterial subpopulations with data pooled from all treatments ($n = 45$). (A) EUB338-detectable cells; (B) BET42a-detectable cells; (C) R-BT065-detectable cells. Full lines depict linear regressions for all data, and the r^2 value shows the coefficient of determination of the regressions. In C, the open symbols are those from bacterivore-free treatments only ($n = 20$) fitted with the dashed regression line; closed symbols are for treatments with the presence of grazers. All regressions are significant ($P < 0.0001$).

targeted by the HGC69a probe (Fig. 4). The cells from this lineage were generally very small (0.054 to 0.085 μm^3), and moreover, in all treatments, they seem to be discriminated against by HNF. The latter finding is consistent with several reports (18, 31) documenting grazing resistance of some actinobacterial phylotypes. This phenomenon could be related to their small cell size but also partially to some size-independent traits, as has been shown for the MWH-Mo1 strain described previously by Hahn et al. (18). The actinobacterial phylotypes in the reservoir were small cells, and their relative abundance was not correlated with %HNA bacteria; they showed growth rates comparable to the average values of the whole bacterioplankton. Thus, while apparently avoided by HNF, the relatively short doubling time (12 to 23 h [Table 1]) gave them a competitive advantage so that they were overrepresented in the presence of grazing. For example, in the heavily grazed bottle <5-μm treatment, they reached as much as 30% of total bacteria (Fig. 4). However, the proportions of *Actinobacteria* showed enhanced variability when <0.8- and <5-μm treatments are compared at time zero (Fig. 4), which could be partially related to the initial sample manipulation via size fractionation.

TABLE 4. Correlation relationships between the proportions of β-*Proteobacteria* (the BET42a probe-detected cells) and of the subcluster of β-*Proteobacteria* (R-BT065 probe-detected cells) as components of the bacterial community versus basic bacterioplankton parameters throughout all treatments[a]

Parameter	% BET42a		% R-BT065	
	Correlation coefficient (*r*)	Significance (*P*)	Correlation coefficient (*r*)	Significance (*P*)
Bacterial abundance	0.615	<0.0001	0.810	<0.0001
Bacterial biomass	0.587	<0.0001	0.753	<0.0001
Bacterial mean cell volume	0.661	<0.0001	0.575	<0.0001
Bacterial production	0.529	0.0004	0.546	0.0002

[a] For calculations, the proportions of FISH-detected bacterial subgroups were expressed as a percentage of total DAPI-stained bacteria. For all analyses, $n = 45$.

This actinobacterial group is considered to be ubiquitous in lakes of various trophic state, size, and geographical location (15, 18, 48, 51). The known or cultivated phylotypes affiliated to the freshwater *Actinobacteria* group are small- to medium-size cells (see, e.g., reference 18), and they seem to be an autochthonous and phylogenetically highly diverse component of freshwater microbial assemblages (50). However, the role of the phylotypes in their habitats remains largely unknown, and even identical actinobacterial sequence types collected from very different locations displayed different growth characteristics when growing in the same medium (18).

To our knowledge, there is no similar study dealing specifically with the ecological role of the lineage detected by the R-BT065 probe (a subcluster of the cosmopolitan cluster *Rhodoferax* sp. BAL47) in a single environment. This group played a main role in the reservoir bacterioplankton (Fig. 4) since its contribution to total bacteria was tightly correlated to most bacterioplankton bulk parameters and responded very clearly to both top-down and bottom-up manipulations (cf. Tables 1 through 4). Apparently, this lineage involves one or more species representing the true example of an opportunistic bacterial strategy in the system. Similar ecological capabilities have been shown for a freshwater lineage beta II in the lake Grösse Fukchskuhle (4) and several groups of marine γ-*Proteobacteria* like *Alteromonas*, *Pseudoalteromonas*, and *Vibrio*, which consistently react to enrichment and grazing in laboratory experiments (2, 9).

In this study, as well as in a previous one (46), the increases in β-proteobacteria (BET42a probe) in the grazer-free treatments were almost completely attributable to fast-growing cells detected by the R-BT065 probe, displaying a conspicuously short doubling time of only 8 to 9 h (Table 1) in the dialysis bag treatments. The cells of this lineage were generally larger (0.09 to 0.22 μm^3) rod-shaped cells of rather uniform morphology, certainly vulnerable to predation, without any obvious trait of morphological grazing resistance behavior (see, e.g., reference 17 for a review). Thus, we suggest that the large growth potential of the members of this lineage is the key factor allowing them to efficiently compensate for grazing losses in the grazer-enhanced treatments and to rapidly overgrow other lineages in grazer-free treatments (Fig. 4).

A growing amount of evidence suggests that the HNA bac-

teria are the most active members of the bacterial community, responsible for the major part of bacterial production (13, 24, 40). However, in natural systems, bacterial production can also be affected by the selective removal of possibly larger dividing and metabolically more active cells (41). Thus, growth of grazers can be initially stimulated by enhanced proportions of larger HNA bacteria (47), but generally, in longer incubations, it yields lower proportions of metabolically active or HNA bacteria (see reference 8 and also cf. Fig. 5). Correspondingly, in our study, the proportions of R-BT065-positive cells tightly correlated with bacterial production, biomass, and %HNA bacteria, with the latter correlation even higher when computed for bacterivore-free treatments only (Fig. 6). However, our data did not show any significant correlation between HNF parameters and %HNA bacteria, while apparently, the intensive HNF grazing markedly decreased the %HNA bacteria by the end of incubation (Fig. 5). A longer experiment would probably have reflected this possibility.

Interestingly, at the beginning of the experiment, when HNF were efficiently top-down controlled during the clear-water phase, proportions of HNA bacteria were very low (~30% of total bacteria) in all treatments. This might suggest that cladoceran filtration (which is responsible for the clear-water phase) removed primarily larger bacteria (20), presumably the pool of HNA bacteria, as HNA bacteria are the largest component of the bacterial community (reference 25 and also see Table 3 in this study). After removing zooplankton from our size-fractionated samples, the initially very small bacterial MCV (~0.08 μm^3) grew markedly together with increasing proportions of HNA and R-BT065-positive bacteria in all treatments. The only exception was bottle <0.8-μm treatment, where MCV increased only slightly while the proportions of R-BT065-positive cells stayed largely invariable (Fig. 4).

More evidence of the high growth and metabolic rates, as well as of ecological flexibility, of R-BT065-positive cells was found in a similar manipulation experiment conducted in the reservoir in May 2003. FISH combined with microautoradiography showed that the cells detected with the R-BT065 probe remained a very important group in the assemblage, and they were the only phylogenetic group that showed a marked increase in the percentage of metabolically active cells ([^{3}H]leucine uptake from ~60 to 95%), even under heavy grazing pressure in <5-μm treatments (K. Horňák, unpublished data).

Overall, when data for all our treatments are pooled, changes in numbers of cells detected with the R-BT065 probe explained ~89% ($r^2 = 0.891$, $P < 0.0001$) of the variability in the numbers of HNA bacteria. This finding suggests an important role of this subgroup in the reservoir bacterioplankton dynamics and underlines the need to further identify the particular members of this small but important lineage whose members were found in various freshwater environments (15, 51). Thus, though we have strong evidence concerning the role of this prominent lineage in overall bacterioplankton dynamics (cf. Fig. 4 and Tables 2 to 4), the relationship with HNA cells is based only on the correlation approach. As yet, there have not been any attempts to identify which groups or species are major component in the fraction of the HNA in freshwaters, in contrast to marine studies (see, e.g., references 25 and 50).

Interestingly, an enrichment experiment with North Sea bacterioplankton (9) and dilution experiments with water from the English Channel (10) clearly documented that manipulating nutrient supply yielded a dramatic community shift with HNA bacteria dominated mainly by opportunistic groups of γ-proteobacteria that usually account for only a minor part of the original nonmanipulated marine samples. In contrast, our typical freshwater group of opportunistic phylotypes belonging to the R-BT065 cluster has always been a very abundant group in the nonmanipulated reservoir bacterioplankton (references 26, 43, and 46 and also see Fig. 4 in this paper).

In this study, we detected a small subcluster of β-proteobacteria of importance in bacterioplankton dynamics in the reservoir. For example, they appear to be responsible for carrying out most of the bacterial production processes. The members of this lineage are extremely fast-growing cells, vulnerable to protist predation without detectable MCV changes in the presence of predators but having the ability to sensitively respond to variable experimental manipulations. Interestingly, while the typical marine opportunistic strategists found among γ-proteobacteria (9) are proportionally less important in natural assemblages, the target for selective HNF predation (2), the members of the R-BT065 cluster, constituted both seasonally as well as spatially a remarkable part of the reservoir bacterioplankton assemblages, accounting for 50 to 100% of the total β-proteobacteria (26, 43, 46). The isolation of the particular members of the R-BT065 cluster and characterization of its phylogenetic diversity are beyond the scope of this study.

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