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Effects of decreased resource availability, protozoan grazing and viral impact on a structure of bacterioplankton assemblage in a canyon-shaped reservoir

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Abstract

We conducted a transplant experiment to elucidate the effects of different levels of grazing pressure, nutrient availability, especially phosphorus, and the impact of viruses on the changes in the structure of bacterioplankton assemblage in a meso-eutrophic reservoir. A sample taken from the nutrient-rich inflow part of the reservoir was size-fractionated and incubated in dialysis bags in both inflow and dam area. The structure of bacterial assemblage was examined by fluorescence in situ hybridization using oligonucleotide probes with different levels of specificity. In terms of the relative proportions of different bacterial groups, we found very few significant changes in the bacterioplankton composition after transplanting the treatments to the nutrient-poor dam area. However, we observed marked shifts in morphology and biomass towards the development of filaments, flocs and "vibrio-like" morphotypes of selected probe-defined groups of bacteria induced by increased grazing pressure. Despite the very high abundances of viruses in all the treatments, their effects on bacterioplankton were rather negligible.

Keywords: Reservoir; Bakterioplankton; In situ hybridization; Protozoan grazing; Viral lysis; Phosphorus

1. Introduction

Planktonic bacteria play an important role in nutrient recycling, organic matter decomposition and biomass formation in aquatic systems, representing thus a relevant link in pelagic microbial food webs. It is generally believed that the growth of planktonic bacteria is regulated mainly by temperature, the amount of available inorganic and organic nutrients ("bottom-up control") and grazing by predators or viral lysis ("top-down control", e.g. [1,2]). Considering bottom-up factors in a typical planktonic system, bacterial growth is not only limited by the supply of autochthonous labile organic carbon substrates produced by phytoplankton, but also by the impact of phosphorus and nitrogen introduced into the system [3]. In addition, bacteria can utilize dissolved organic matter (DOM) of allochthonous origin coming from the terrestrial environment and may exhibit different growth rates in various high and low molecular weight components of DOM [4]. Moreover, within the whole bacterial community there are bacterial populations differing strongly in their growth response to changing resource availability and food web structure [5].

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Heterotrophic nanoflagellates (HNF) and ciliates (CIL) are considered to be the most efficient bacterial consumers [6]. Protozoan grazing pressure induces changes in the abundance, size structure, activity and composition of bacterioplankton assemblages [7]. Protozoan predators affect planktonic bacteria by preying on the larger cells [8], on the metabolically most active cells [9] or specifically on a given bacterial group [10]. Protistan bacterivory is also associated with the development of various grazing-resistant forms, mainly filaments and flocs [11,12].

Another source of bacterial mortality is viral lysis, which can remove a comparable proportion of bacterial production (BP) to that removed by grazing [1]. It has been suggested that the impact of viruses on bacterial community composition (BCC) seems to be more pronounced with increasing system productivity [13]. Viral production can be also enhanced after enrichment with inorganic and organic nutrients [14] and the possible synergic effect between the increased protozoan pressure and viral activity has already been suggested [15,16].

In the present experiment, we focused especially on the effects of different top-down and bottom-up factors regulating bacterioplankton community composition and dynamics. Size-fractionation of samples together with bottom-up manipulations via transplantation of the samples into areas with different nutrient availability, within a canyon-shaped reservoir [17] can specifically contribute to the elucidation of the effects of top-down and bottom-up regulation of bacterioplankton. A canyon-shaped reservoir offers an ideal model system to study the relationships within the microbial food webs, owing to its very high level of spatial and longitudinal heterogenity of major limnological parameters [18].

It is one of the major topics of current aquatic microbial ecology to examine the stability of BCC in relation to changing food web structure [7,19,20]. Changes in BCC are mostly monitored by fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes [21] with different levels of specificity, which has become one of the commonly used methods to study the phylogenetic composition of bacterioplankton assemblages.

Based upon the results obtained from the previous experiment conducted in the Římov reservoir, exploring the dialysis bag technique [22], we hypothesized that the BCC is determined mainly by the predation pressure (top-down control) on samples transplanted from the nutrient-rich to the more nutrient-limited dam area of the reservoir, while the effect of available nutrients (bottom-up control) on BCC prevails in the inflow part of the reservoir. Moreover, we focused on the simultaneous evaluation of the effects of protozoan grazing and the impact of viruses on bacterioplankton assemblage. We expected the more pronounced effect of viral-induced bacterial mortality (VIBM) in the nutrient-rich inflow part and in the <5 μm treatments with enhanced grazing pressure [16].

2. Materials and methods

2.1. Study site and experimental setup

The experiment was conducted in the canyon-shaped Římov reservoir (South Bohemia; 470 m a.s.l.; area, 2.06 km^2; volume, 34.5×10^6 m^3; length, 13.5 km; max. depth, 43 m; mean depth, 16.5 m; mean retention time, 100 d; dimictic, meso-eutrophic) during the clear-water phase (21 May–25 May 2001). We chose two experimental sites in the reservoir strongly differing in the content of nutrients and perhaps also in the nature and amount of organic C resources and, moderately, also in water temperature (see Section 3). The first experimental site was located in the river inflow to the reservoir just above the plunge point (=station "RIVER"), the second close to the dam of the reservoir (=station "DAM").

On 21 May 2001, water sample was collected from the riverine part (RIVER) of the reservoir with a 2-l Friedinger sampler from the surface level (depth ~0.5 m) into the plastic container. A total volume of 40 l of sample was collected. The water sample was immediately size-fractionated by subsequent filtering of the water through 5- and 0.8-μm pore-size white Poretics membrane filters (diameter, 47 mm; OSMONICS Inc., Livermore, USA) into following size fractions: <5 μm treatment – containing bacteria, viruses, HNF and small algae only; <0.8 μm treatment – containing only bacteria and viruses. The changes in the dynamics of microbes in the latter two size fractions were compared to those occurring in unfiltered treatment – with all bacterial and HNF consumers present. The water size fractions were placed into dialysis bags (a total of 12 bags; each containing ~2 l of a water sample; diameter of dialysis tubes, 75 mm; molecular weight cutoff, 12,000–16,000 Da [Poly Labo, Switzerland]). Before use, the dialysis bags were boiled and rinsed three times in hot redistilled water. The filled bags were incubated in the reservoir in the open plastic holders in a horizontal position at a depth of 0.5 m (for more details see e.g. [15]). For analysis of the microbial parameters, subsamples of 300–400 ml were taken from each dialysis bag at intervals of 0, 24, 48, 72, and 96 h (t_0, t_{24}, t_{48}, t_{72}, and t_{96}). The three different fractions of the water sample taken from the riverine part were incubated in dialysis bags at the sampling site (=RIVER), and in parallel transplanted at the dam area (=DAM). Thus, we incubated six dialysis bags (<0.8 μm, <5 μm, and unfiltered treatment in duplicates) at each experimental site.

2.2. Bacterial abundance and biomass

Samples were fixed with prefiltered formaldehyde (2% vol/vol final concentration), 1–2 ml subsamples were stained with fluorochrome 4,6-diamino-2-phenylindole (DAPI; 0.2% wt/vol final concentration) and filtered through 0.2 μm black membrane Poretics filters (25 mm diameter, OSMONIC INC., Livermore, USA). Samples were counted (at least 500 bacteria per sample) using epifluorescence microscopy (Olympus BX-60). Between 400 and 600 cells were recorded at a magnification of 1000× with an analog monochrome CCD camera (Cohu Inc., San Diego, USA) and processed with the semiautomatic image analysis system LUCIA D (Lucia 3.52, resolution 750×520 pixels, 256 gray-levels, Laboratory Imaging, Prague, Czech Republic), for details see [23]. Bacterial biomass was calculated according to the allometric relationship between cell volume and carbon content reported by [24]. Cells longer than 4 μm and bacterial aggregates >4 μm (sized across) were considered to be resistant against grazing by most bacterivorous protists [12,20].

2.3. Bacterial production

Bacterial production was determined by the thymidine incorporation method described by [25]. To each duplicate 5-ml subsample, 10 nmol l^{-1} of [methyl-^{3}H]-thymidine (Amersham) was added. Samples were incubated at in situ temperature in the dark for 30 min, then preserved with neutral buffered formaldehyde (2% vol/vol final concentration), filtered through 0.2-μm membrane Poretics filters (25 mm diameter, OSMONIC INC., Livermore, USA) and rinsed 10 times with 1 ml of ice-cold 5% TCA [20]. Replicate blanks prefixed with 2% formaldehyde were processed in parallel. The data on bacterial cell number increase from replicated <0.8 μm treatments incubated in situ at both the RIVER and DAM stations were used to establish an empirical conversion factor (ECF) between the thymidine incorporation rate and the bacterial cell production rate. ECFs 1.57×10^{18} and 1.81×10^{18} cells mol^{-1} thymidine (for station RIVER and DAM, respectively) were calculated using a modified derivative method described by [26].

2.4. Bacterial community composition

We analyzed changes in BCC by FISH with group-specific rRNA-targeted oligonucleotide probes on membrane filters [21]. Samples prefixed with alkaline Lugol's solution were fixed with formaldehyde (2% vol/vol final concentration) for 1 h and then decolorized with several drops of a 3% solution of sodium thiosulfate [27]. Afterwards, each duplicate sample between 5 and 25 ml was filtered at low pressure (⩽15 kPa) through 0.2-μm pore-size white Poretics membrane filters (diameter, 47 mm), which were supported by a 1-μm pore-size Poretics membrane filter. Filters with concentrated bacteria were rinsed with 2×15 ml redistilled water and stored at −20 °C until further processing. We used the following oligonucleotide probes (Interactiva, Ulm, Germany) with different levels of specificity: the domain *Bacteria* (EUB) targeted by EUB338, the α-, β-, γ-subclasses of the class *Proteobacteria* (ALF, BET, GAM) targeted by ALF968, BET42a, GAM42a, the *Cytophaga-Flavobacterium* group (CF) targeted by CF319a, two more specific fylogenetic lineages (a small subcluster of β-*Proteobacteria*, R-BT, and the *Flectobacillus* cluster, R-FL) targeted by probes R-BT065 and R-FL615, respectively (details in [15]), and the group *Actinobacteria* (high G + C Gram positive bacteria) targeted by HGC69a [28]. All probes were fluorescently labelled with the indocarbocyanine dye Cy3 (Interactiva, Ulm, Germany). After hybridization, the filter sections were stained with 4,6-diamino-2-phenylindole (DAPI) and rinsed in redistilled water and 96% ethanol, respectively. Air-dried filter sections were enumerated by epifluorescence microscopy (PROVIS AX 70, Olympus) using a 4:1 mixture of Citifluor and VectaShield mounting medium. At least 500 DAPI stained cells were counted per sample.

2.5. Protozoan abundance and grazing rate

Protozoan grazing on bacterioplankton was estimated by a technique of fluorescently labelled bacterioplankton (FLB [27]). FLBs were prepared from the concentrated bacterioplankton sample from the Římov reservoir and stained with fluorochrome 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein (DTAF). Rates of FLB uptake were estimated in the water sample from each dialysis bag with the presence of bacterivores, i.e. <5 μm and unfiltered treatment at each time point. All samples (100 ml) with FLBs (5–15% of total bacterial abundance) were incubated for up to 30 min at in situ temperature after the addition of tracer. Subsamples taken at 5, 10 and 30 min were prefixed with 0.5% alkaline Lugol's solution, fixed with formaldehyde (2% vol/vol final concentration) and finally cleared with several drops of a 3% solution of sodium thiosulfate [27]. At least 50 DAPI-stained CIL and 100 HNF were inspected and enumerated per sample, for details of the grazing experiments and protozoan counting see e.g. [22]. To estimate the total grazing rate (TGR), in situ abundances of CIL and HNF were multiplied by their cell-specific grazing rates.

2.6. Enumeration of viruses

Samples were fixed with formaldehyde (2% vol/vol final concentration). Virus-like particles (VLPs) were counted within 10 days after sample collection according

to the protocol of [29] using epifluorescence microscopy and the fluorochrome YOPRO-1. This protocol, which is a modification of the one originally proposed by [30], reduces the time needed to stain viruses from 48 h to 4 min, works with samples fixed by aldehydes, and produces reliable counts of free viruses in aquatic ecosystems [31]. A stock solution of Yo-Pro-1 (Molecular Probes Europe, Leiden, The Netherlands) was diluted to 50 μM in an aqueous solution of 2 mM NaCN. VLPs and bacteria in 1–3 ml of reservoir samples were gently filtered (<15 kPa vacuum) onto 0.02 μm pore-size Al_2O_3 Anodisc filters (Whatman) and rinsed three times by filtering with 500 μl deionized-distilled water (DDW). Filters were put sample side up on 80 μl drops of Yo-Pro 1 in a Petri dish, that was placed in a cardboard container to protect it from light, and irradiated in a domestic microwave oven equipped with a turntable for no longer than 4 min at a low-intermediate power level (~400 W). The heated Petri dish was allowed to cool for about 10 min afterwards, the filters were replaced on the filtering support and rinsed three times with 800 μl portions of DDW. The filters were transferred to glass slides, covered with a single drop of a solution containing 50% glycerol, 50% phosphate buffered saline (0.05 M Na_2HPO_4, 0.85% NaCl, pH 7.5), and 0.1% *p*-phenylenediamine (freshly made daily from a frozen 10% aqueous stock solution; Sigma) on 25 mm square coverslips. This mountant minimised fading [32]. All working solutions were filter sterilized immediately before use with Anotop 10 units (Whatman) equipped with 0.02 μm pore-sized inorganic membranes and sterile syringes. The VLPs were counted by using an Olympus HB2 microscope equipped with a 100/1.25 Neofluar objective lens and a wide blue filter set. The size, the distinctive shape and the very much brighter fluorescence of bacteria clearly distinguished these particles from viruses. Triplicate counts of subsamples yielded SDs of <5%.

2.7. Phage-infected bacteria and subsequent mortality

In formalin-fixed samples, bacteria contained in 8 ml subsamples were harvested by ultracentrifugation onto grids (400 mesh NI electron microscope grids with a carbon-coated Formvar film) by using a Centrikon TST 41.14 Swing-Out-Rotor run at 70,000*g* for 20 min at 4 °C [33,34]. Each grid was then stained for 30 s with uranyl acetate (2% wt/wt) and examined in a JEOL 1200EX transmission electron microscope operated at 80 kV at a magnification of 40,000×. Because of the high acceleration voltage (80 kV) used in this study, we were able to identify bacterial cells containing mature phages. A cell was considered infected when the phages inside could be clearly recognized on the basis of shape and size. At least 600 bacterial cells were inspected per sample to determine an infection rate or frequency of visibly infected cells (FVIC). To estimate the VIBM, the frequency of visibly infected cells (FVIC, as a percent) was first related to the frequency of infected cells (FIC) as follows: FIC = 9.524 FVIC-3.256 [35]. FIC was then converted to VIBM according to [36]: VIBM = (FIC + 0.6 FIC^2)/(1–1.2 FIC). In a steady system, bacterial mortality due to viral lysis matches the bacterial production, which is removed by lysis [37]. Thus, multiplying the lysed bacterial production by the burst size (i.e. the number of viruses produced in a cell) yielded viral production [38].

2.8. Chemical analysis

The concentration of dissolved organic carbon (DOC) was determined using Shimadzu TOC-5000A analyzer. Concentrations of total phosphorus (TP) and dissolved reactive phosphorus (DRP) were measured according to the spectrophotometrical method reported by [39].

2.9. Zooplankton composition

Zooplankton was concentrated through 40 μm mesh-size filter from ~10 l of raw water sample at the beginning of the experiment, fixed with formaldehyde (4% vol/vol final concentration) and analyzed and counted from several subsamples according to [40]. The same procedure has been used to analyze the zooplankton composition from the water remaining in the unfiltered treatments incubated in dialysis bags (~1000 ml in both RIVER and DAM) at the end of the experiment (t_{96}).

2.10. Statistical analysis

At first, data on the relative proportions of different probe-defined bacterial groups were normalized using arc-sin transformation. A two-way analysis of variance (ANOVA) was applied to test the changes in the contributions of the probe-defined bacterial groups between incubation site (RIVER vs DAM) and size-fraction treatments (<0.8 μm, <5 μm, unfiltered) at intervals of 24, 48, 72 and 96 h. One-way ANOVAs were used to test for the changes in the proportions within each probe-defined bacterial group between the treatments at either RIVER or DAM site apart. Then, in significant ANOVAs Tukey's Multiple Comparison post test was applied for pair-wise comparisons between each size-fraction treatments. All statistics was performed using GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA, USA).

3. Results

In the canyon-shaped Římov reservoir, a marked longitudinal gradient of major limnological parameters be-

tween the riverine and lacustrine part of the reservoir was established during the experimental period. At the station RIVER, the water temperature was only 9 °C at the starting point of the experiment and afterwards it settled to 12–13 °C, while at the station DAM it was ~17 °C during the whole experiment. Remarkable differences were found in the concentration of chemical parameters: The station RIVER was rich in total (TP, 76 $\mu g\, l^{-1}$) and dissolved reactive phosphorus (DRP, 32 $\mu g\, l^{-1}$), while these parameters were several times lower at the station DAM (16 and 4 $\mu g\, l^{-1}$, respectively). In contrast, no marked changes occurred in concentrations of dissolved organic carbon (DOC), which was commonly 4.5 $mg\, l^{-1}$ in both the RIVER and DAM areas. The concentration of chlorophyll *a* was not specifically determined in the experiment, but ~7.5 $mg\, l^{-1}$ of chl *a* was found in the dam area on 29 May 2001 (unpublished data).

There were only very few individuals of small Cladocerans and Rotifers present in the water sample taken directly from the river inflow at the experiment start. Only a few Rotifers in the unfiltered DAM treatment and practically no zooplankters were found in unfiltered RIVER treatment from the remaining water sample in dialysis bags at the end of the experiment (t_{96}). Thus, the impact of metazooplankton grazing on our unfiltered treatments was most likely negligible.

3.1. Bacterial and protozoan dynamics

Across all treatments, during the first two days of incubation, the bacterial abundance increased from $\sim 1.4 \times 10^6$ to $10–16 \times 10^6$ ml^{-1} at 48 h (Fig. 1). After the second day of incubation a rapid increase in bacterial abundance continued only in the RIVER <0.8 μm treatment up to $\sim 26.6 \times 10^6$ ml^{-1}. In all remaining treatments bacterial abundance decreased due to heavy grazing (especially at the samples transplanted to DAM) or remained stable (RIVER-incubated unfiltered treatment). Large changes in bacterial mean cell volume (MCV) occurred especially in the <5 μm and partly also in the unfiltered treatments during the last two days of incubation (t_{48}–t_{96}). Bacterial MCV markedly increased in the DAM <5 μm and unfiltered treatments while in corresponding RIVER treatments it increased only slightly (Fig. 1). In RIVER-incubated treatments the increase of bacterial production (BP) was slightly slower, but continuous, so that it achieved higher values at the end of the experiment (<5 μm and unfiltered treatments) than in corresponding DAM treatments (Fig. 2). BP had a comparable increasing pattern in both <0.8 μm treatments until 72 h. Unfortunately, DAM-incubated <0.8 μm treatment has been influenced by protozoan contamination during the last day of experiment. Between t_{48}–t_{72} BP reached the maximal values of $12.5–15.5 \times 10^6$ $ml^{-1}\, day^{-1}$ in DAM-incubated treatments and then gradually decreased (see namely the <5 μm treatment in Fig. 2).

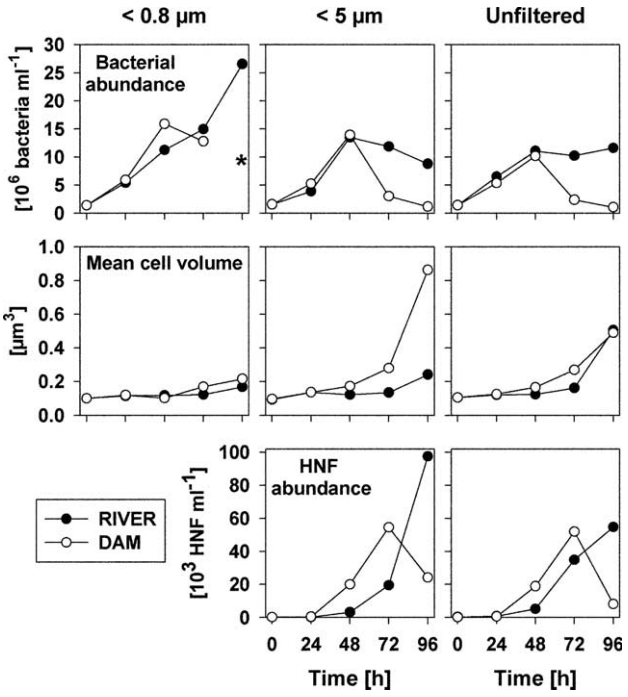

Fig. 1. Changes in abundances and MCVs of bacteria and abundances of heterotrophic nannoflagellates (HNF) in different size treatments (<0.8 μm, <5 μm, and unfiltered) incubated at the nutrient-rich inflow area (RIVER) and in parallel transplanted to the nutrient-poor area (DAM). Bacterial abundance in upper panels, bacterial MCV in middle panels, abundance of HNF in lower panels. Values are means for two replicate treatments. * value influenced by protozoan contamination.

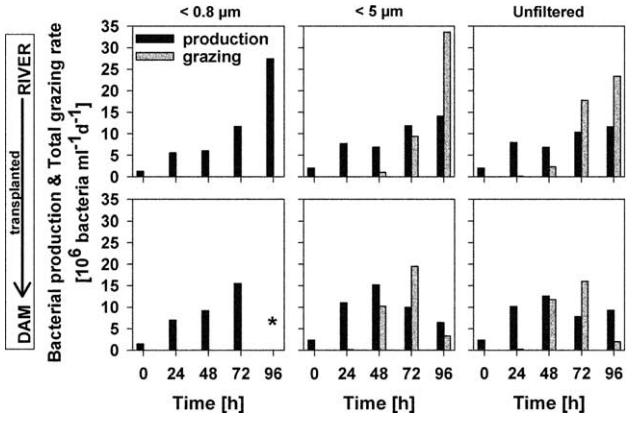

Fig. 2. Changes in bacterial production and total protozoan grazing rate in different size treatments (<0.8 μm, <5 μm, and unfiltered) incubated at the nutrient-rich inflow part (RIVER) and in parallel transplanted to the nutrient-poor dam area (DAM). Values are means for two replicate treatments. * value influenced by protozoan contamination.

We also calculated the net doubling times for each probe-defined group of bacteria in comparison to the whole bacterial community in the <0.8 μm treatments

and HNF in the <5 μm and unfiltered treatments (data summarized in Table 1). The net doubling times calculated for each group of organisms varied between 11 and 16 h. The shortest doubling time was calculated for the BET. However, most likely due to the large difference in water temperature, we did not find any significant difference in net doubling times between RIVER- and DAM-incubated <0.8 μm treatments.

Marked changes occurred in the abundances of heterotrophic nanoflagellates (HNF) in all <5 μm and unfiltered treatments (Fig. 1). The abundance of HNF continuously increased until the end of the experiment in both RIVER-incubated treatments (Fig. 1). However, in the corresponding DAM treatments the increase stopped by the third day of incubation (t_{72}), and it was followed by a dramatic decrease especially in the DAM unfiltered treatment. This marked decrease in HNF abundance apparently reflected the sharp drop in bacterial prey abundance (Fig. 1). Between t_0 and t_{72}, there were similar trends in TGR found in RIVER and DAM samples in both <5 μm and unfiltered treatments (Fig. 2). In both RIVER treatments TGR increased rapidly namely during the last two days of experiment, while in the DAM treatments TGR decreased during the last day and the grazing impact on bacteria was ~20 times lower then in the corresponding RIVER treatments. Overall, across all treatments, the observed pattern in TGR tightly followed the changes in bacterial abundance.

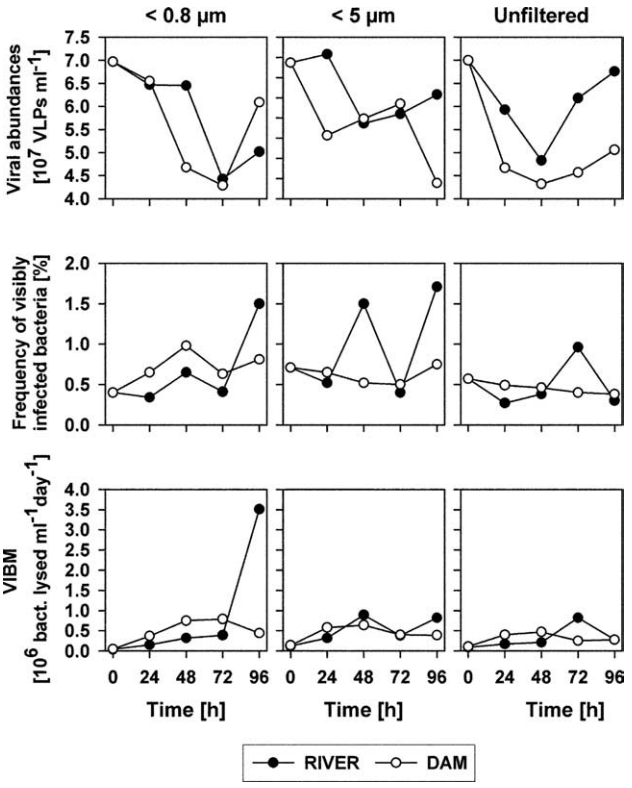

Fig. 3. Changes in total viral abundance, FVIB, and VIBM in different size treatments (<0.8 μm, <5 μm, and unfiltered) incubated at the nutrient-rich part (RIVER) and in parallel transplanted to the nutrient-poor dam area (DAM). Values are means for two replicate treatments.

3.2. Viral impact

There were no clear temporal changes in terms of viral abundance, the frequency of visibly infected bacteria (FVIB), nor viral impact during the experiment (Fig. 3). Although the viral abundance, FVIB and viral induced bacterial mortality (VIBM) were on average higher in the station RIVER, these differences were not significant. The abundance of VLPs varied between 4 and 8×10^7 ml^{-1} showing ambiguous changes in all RIVER and DAM treatments. Similarly, FVIB varied between 0.3% and 1.7% of total bacteria without any clear trend during the whole experiment. VIBM was negligible or varied between 0.5 and $\sim 0.8 \times 10^6$ bacteria lysed ml^{-1} day^{-1} in all treatments. Only in RIVER <0.8 μm treatment, there was a strong increase in viral impact ($\sim 3.5 \times 10^6$ bacteria lysed ml^{-1} day^{-1}) at 96 h.

3.3. Bacterial community composition

Temporal changes in BCC determined by FISH are shown in Fig. 4. In general, in terms of the relative pro-

Table 1
Net doubling times of various probe-defined groups of bacteria and bacterioplankton assemblage in the <0.8 μm treatment, and HNF in <5 μm and unfiltered treatment incubated at the nutrient-rich inflow part (RIVER) and the nutrient-poor area (DAM)

Target group	Doubling time (h)		Interval (h)	Treatment
	Incubation at RIVER	Incubation at DAM		
Bacterioplankton	14 ± 0.6	16 ± 0.9	0–48	<0.8 μm
BET	11 ± 0.7	11 ± 1.1	0–48	<0.8 μm
R-BT	13 ± 1.2	12 ± 1.3	0–48	<0.8 μm
CF	13 ± 2.2	15 ± 1.0	0–48	<0.8 μm
GAM	15 ± 0.7	16 ± 1.4	0–48	<0.8 μm
ACT	15 ± 0.3	15 ± 1.5	0–48	<0.8 μm
HNF	14 ± 0.6	14 ± 0.6	48–72	<5 μm
HNF	14 ± 0.6	14 ± 0.6	48–72	Unfiltered

The values are means ± range of two replicate treatments. Doubling times were calculated for the period with exponential growth of targeted groups.

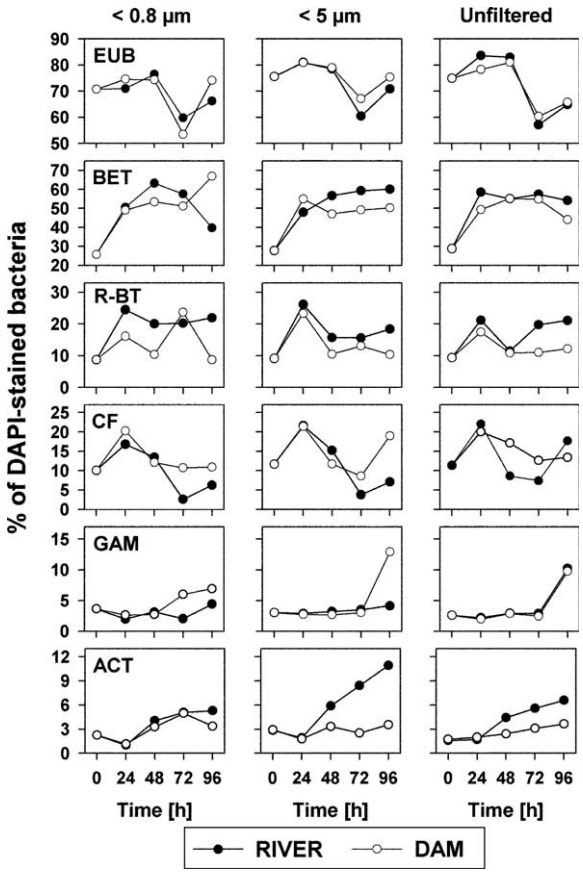

Fig. 4. Comparison of bacterioplankton phylogenetic composition monitored by FISH in different size treatments (<0.8 µm, <5 µm, and unfiltered) incubated at the nutrient-rich inflow area (RIVER) and in parallel transplanted to the nutrient-poor area (DAM). Bacteria were classified using oligonucleotide probes affiliated to the following bacterial groups: *Bacteria* (EUB), β-*Proteobacteria* (BET), R-BT065 cluster (R-BT), *Cytophaga-Flavobacterium* (CF), γ-*Proteobacteria* (GAM), *Actinobacteria* (ACT). Values are means for two replicate treatments.

Table 2
Results of two-way ANOVA (*F*-values) applied to test the changes in the composition of bacterial assemblage induced by transplanting the treatments from the nutrient-rich station (RIVER) to the nutrient-poor area (DAM) and by filtering the water sample to different size-fractions (data not shown) at intervals of t_{24}, t_{48}, t_{72} and t_{96}

Probe	t_{24}	t_{48}	t_{72}	t_{96}
RIVER samples transplanted to the DAM (*F-values*)				
EUB	0.02	0.01	1.8	0.72
BET	0.06	5.24	11.59*	0.24
R-BT	33.11**	inter	3.6	45.23***
CF	2.68	inter	inter	6.68*
GAM	0.69	1.14	16**	3.28
ACT	0.04	10.65*	3.82	14.02**

Tested were changes in the relative proportions (as % of total DAPI-stained bacterial cells) of six different probe-defined phylogenetic groups of bacteria affiliated to the groups of EUB, BET, R-BT, CF, GAM and ACT (see Fig. 4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, inter – significant interaction between the effects of longitudinal transfer and size-fractionating the water sample.

portions of large phylogenetic groups of bacteria, very few significant changes occurred in BCC between the RIVER- and DAM-incubated treatments (see Table 2). The hybridization efficiency, determined as the portion of bacteria hybridized with the general probe EUB338 for *Bacteria* (EUB), varied between 55% and 85% of the DAPI stained cells in all treatments, with a marked decline during the third day of the experiment.

Bacteria affiliated to the subclass of α-*Proteobacteria* (ALF) represented a negligible portion of the overall community, being under the reliable detection limit of FISH (<1% of DAPI stained cells during the whole experiment, data not shown).

The most of bacteria were affiliated to β-*Proteobacteria* (BET). Across all treatments, BET showed a steep increase at the beginning, accounting for 50–65% of total bacteria after the first 24 h of incubation till the end of the experiment (Fig. 4), with generally very similar pattern of changes found in both RIVER- and DAM-incubated treatments.

The narrower cluster of R-BT (a subgroup of the subclass β-*Proteobacteria*) showed quite similar temporal changes in its contributions in <5 µm and unfiltered treatments. Consistently, the initial increase was followed by a decline (Fig. 4). Different patterns of R-BT proportion occurred in <0.8 µm size fractions. In RIVER <0.8 µm treatment the proportion of R-BT cluster increased ~twice and remained almost constant until the end of the experiment. There was no clear trend of

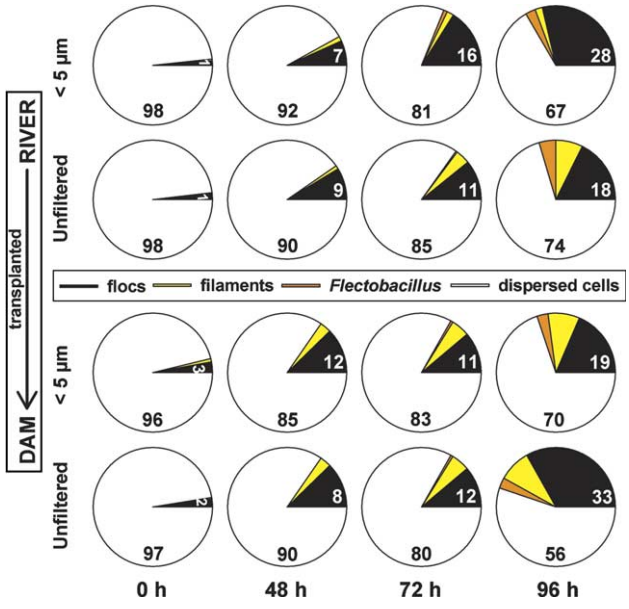

Fig. 5. Development of grazing-resistant morphotypes (flocs and filaments) in comparison to freely dispersed bacteria in <5 µm and unfiltered treatments incubated at the nutrient-rich inflow area (RIVER) and in parallel transplanted to the nutrient-poor part (DAM). Numbers included in each graph indicate the relative proportions of floc-forming and dispersed bacterial cells at 0, 48, 72 and 96 h. Values are means for two replicate treatments.

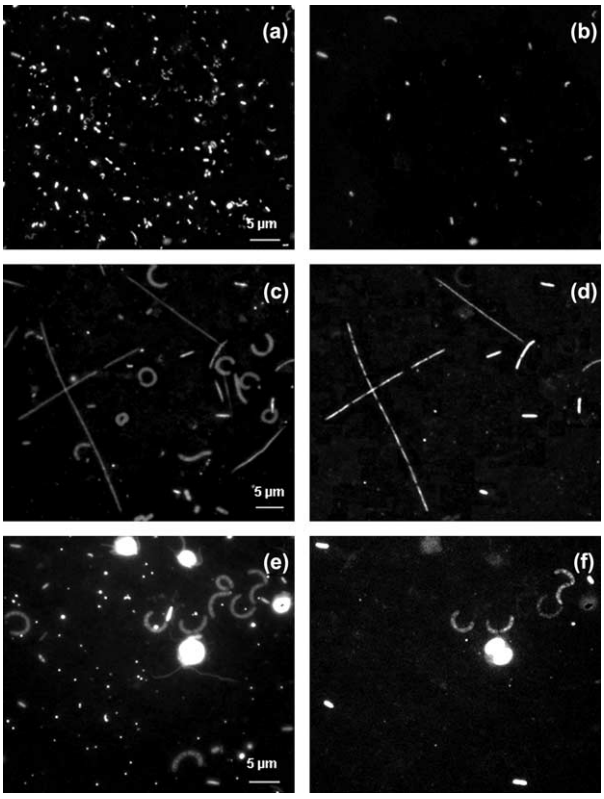

Fig. 6. Images of various groups of bacteria differing in their morphology. Left column: DAPI-stained bacteria, right column: Cy3-labeled bacteria. (a, b) Typical morphology of bacterioplankton assemblage (β-*Proteobacteria* is shown) at the beginning of the experiment. (c, d) Long filaments and rods affiliated to the *Cytophaga-Flavobacterium* group at the nutrient-rich area (RIVER) in the <5 μm treatment at the end of the experiment. Filaments composed of a chain of cells (see (d)) were also affiliated to the specific *Flectobacillus* cluster. (e, f) Rods and "vibrio-like" cells affiliated to the γ-*Proteobacteria* group at the nutrient-rich area (RIVER) in the <5 μm treatment at the end of the experiment.

R-BT proportion found in transplanted DAM <0.8 μm treatment.

The proportion of bacteria belonging to the *Cytophaga–Flavobacterium* (CF) roughly doubled within the first 24 h. Afterwards, there was a common marked decline in proportion of CF group to <5% in RIVER <0.8 μm and <5 μm treatments at t_{72}. Finally, in the last day of incubation the CF group increased again especially in RIVER <5 μm and unfiltered treatments (Fig. 4). A significant proportion (50–80%) of total filaments >10 μm long were affiliated to CF in <5 μm and unfiltered treatments at the end of the experiment. Moreover, 50–60% of total filaments in RIVER and ~25% in DAM (Fig. 5) were affiliated to the narrow *Flectobacillus* cluster (R-FL) that forms filaments of typical morphology composed of a chain of cells (see Fig. 6(d)).

Members of γ-*Proteobacteria* (GAM) mostly contributed negligibly to total bacterial cell numbers within the whole experiment except for the increase during the last day (>10%) in DAM <5 μm and both RIVER and DAM unfiltered treatments (Fig. 4). Interestingly, a marked shift in cell size, morphology and C content of GAM occurred in both <5 μm treatments during the experiment (Table 3). At the beginning, GAM consisted of small, single rods (size 1 μm long, MCV 0.1 μm^3, 22 fg C $cell^{-1}$), while these parameters increased after 2 days (size 2.2 μm, MCV 0.31 μm^3, 50 fg C $cell^{-1}$). At the end, GAM were mainly composed of large, "vibrio-like" cells (size 6.7 μm, MCV 1.24 μm^3, 129 fg C $cell^{-1}$, see Fig. 6(f)). Thus, while the shift in the relative proportions of GAM was only by a factor ~3, the same shift expressed in the biomass of GAM at t_{96} was an increase of more than one order of magnitude.

The relative proportion of *Actinobacteria* (ACT) did not usually exceed 5% (Fig. 4). However, the proportion of ACT continuously increased in unfiltered treatments but it only achieved >10% in the RIVER-incubated <5 μm treatment by the end of the experiment. In comparison to bacterial cells detected by other probes used in the experiment, members of ACT usually appeared as the tiniest cells with a very small MCV (<0.03 μm^3), so that their contribution to total bacterial biomass was quite negligible.

3.4. Grazing-resistant morphotypes

A continuous increase in the proportion of bacterial flocs and filaments has been observed in all <5 μm and unfiltered treatments (Fig. 5). In general, floc-forming cells predominated compared to filaments during the second half of the experiment. At the beginning of the experiment, bacterial grazing-resistant morphotypes were very rare and almost all bacteria were small freely dispersed cells. In contrast, during the last day between ~25% and 45% of total bacteria were aggregated in

Table 3
Characteristics of γ-*Proteobacteria* in different treatments (<0.8 μm, <5 μm, and unfiltered) at intervals of 0, 48, and 96 h

Treatment	0 h	48 h			96 h		
	all	<0.8 μm	<5 μm	Unfiltered	<0.8 μm	<5 μm	Unfiltered
Size (μm)	1	1.6	2.2	1.7	2.2	6.7	2.2
MCV (μm^3)	0.1	0.14	0.31	0.19	0.17	1.24	0.2
C content (fg C $cell^{-1}$)	22	28	50	34	33	129	36

Average cell size, MCV, and C content are shown. Values are means for >50 cells measured per treatment.

either flocs or formed long filaments. The majority of filaments were affiliated to the CF group and to the *Flectobacillus* cluster (R-FL) specifically (see Figs. 5 and 6(d)). Generally, the largest portions of grazing-resistant morphotypes were found in both DAM-incubated treatments (32–45%). Slightly lower proportions were found in both RIVER-incubated treatments at the end of the experiment.

4. Discussion

In this manipulation experiment we simultaneously combined "top-down" and "bottom-up" factors regulating the BCC and dynamics as specifically determined by means of FISH. We built on knowledge from the previous manipulation experiment conducted in the Římov reservoir, where the water sample was transplanted from the dam area to its inflow part, i.e. from a nutrient poor to a nutrient-rich environment (for details see [22]). In contrast, in this experiment (using the same size-fractionation approach), water samples were transplanted from the nutrient-rich inflow part to the nutrient-limited dam area of the Římov reservoir. Thus, while applying an identical design while transplanting the samples, but inversely oriented in terms of the nutrient gradient, we intended to elucidate the influence of available nutrient supply, predation and viral impact on BCC, when supposedly nutrient-saturated microbial communities were transplanted into a much more nutrient-limited environment.

A very similar pattern of bacterial abundance and production was found in both RIVER and DAM <0.8 μm treatments despite the distinct concentrations of phosphorus in both environments. This finding is very different from the trends described in the previous experiment, when nutrient-limited microbial communities were transplanted into a P-rich part of the reservoir [22]. The present data may suggest that bacteria originally growing in the resource-rich inflow area were not simply limited by nutrients after transfer to the more resource-limited DAM area. We argue that one of the reasons could be that the bacterial cells carried a sufficient amount of storage material, in the form of nutrients and perhaps organic C resources, in their biomass, supporting thus their growth for a certain time period even in this more resource-limited DAM area. Moreover, 4 μg of DRP found in the station DAM may not be a limiting amount of P for bacteria [41]. Thus, similar BCC could sustain longer (Fig. 4) even under markedly changed nutrient conditions.

In fact, the bacterial assemblage was transplanted together with the nutrient-rich river water in dialysis bags and bacteria could go on for a while in consuming the nutrients from the dialysis bag content. However, the content of the dialysis bags was most likely diluted and finally levelled off with the nutrient concentration in the ambient water through its penetration in the nutrient-limited dam area at the latest within the first 12–15 h of incubation (experimentally tested, data not shown). Thus, bacterioplankton assemblage might be influenced by the nutrient-rich water during only the first day of incubation.

After transplanting the samples downstream, the relatively fast bacterial growth in the DAM-incubated treatments could result both from the supposed bacterial storage material brought from the river inflow, and also from the significantly higher temperature at the dam area (4–8 °C). Moreover, this combined effect could also explain the almost identical net doubling times calculated for both RIVER- and DAM-incubated treatments (see Table 1). Overall, it seems likely that the higher P-availability in the station RIVER was counterbalanced by the lower growth rate of bacteria in the markedly colder water.

In both treatments with different levels of grazing (<5 μm, unfiltered), bacterial predators efficiently controlled the bacterial abundance, mainly during the second half of experiment, when the grazing pressure even exceeded the bacterial production, as previously described also in [15,22]. Bacterial consumers decreased bacterial abundance, in both <5 μm and unfiltered treatments transplanted to the DAM area, back to their initial abundances, while in the parallel RIVER-incubated treatments bacterial abundance was approximately same or decreased only slightly despite the much higher levels of bacterivory. This suggests that even heavily grazed bacterial assemblages could maintain their abundance by balancing their grazing losses through enhanced growth rate in this nutrient-rich environment. The ability of the major phylogenetic groups of bacteria to outgrow the strong grazing pressure could also have contributed to the relative stability of BCC even in <5 μm and unfiltered treatments [10,22]. The difference in nutrient availability between RIVER and DAM areas can also explain why, in the DAM-incubated treatments, growth did not fully balance grazing-induced bacterial mortality, yielding the observed decreases in bacterial abundances in the DAM <5 and unfiltered treatments (Fig. 1).

4.1. Viruses

Viruses were responsible for 3–12% of daily removed bacterial production in different treatments with maximal impact in P-rich RIVER-incubated samples (Fig. 3). We assumed that the viral activity could be enhanced by the increased grazing pressure especially in <5 μm treatments via grazing-induced changes in BCC or by the higher concentration of phosphorus in the nutrient-rich inflow area [16]. However, the expected increase in viral activity in parallel with enhanced grazing

pressure described previously was not confirmed [22] as holds true for the rather negligible BCC shifts observed at both, differentially P-limited experimental sites. Across all treatments, the abundance of viruses ranged between 3 and 8×10^7 VLPs ml^{-1} with no clear pattern, and were comparable to those found in previous experiments [15,16]. However, in the present study we found significantly lower FVIB cells. Moreover, we did not observe any significant differences in viral abundance, FVIB or viral impact between RIVER- and DAM-incubated samples and different size-fractions. Another expected trend (cf. [16]) between the viral activity and the concentration of available phosphorus was not fully confirmed, though we found slightly higher values of viral parameters at the nutrient-rich station RIVER, where the bacterioplankton was probably more influenced by viruses than at the station DAM.

4.2. Bacterial community composition

At the beginning of the experiment, using FISH with rRNA-targeted oligonucleotide probes with different levels of specificity, BCC was in principle typical for the clear-water phase in the Římov reservoir and basically comparable to that found in the previous manipulation experiments [15,22]. However, in the present experiment we found a higher proportion of EUB, but lower proportion of CF group. The higher proportion of EUB could be related to the generally higher metabolic activity of bacterial cells induced by the higher nutrient availability in the water used for the experiment, as it was originally from the inflow area. Faster-growing or highly active cells harbor a higher rRNA content in their biomass, and hence bind proportionately more probe molecules, resulting in a stronger fluorescent signal of hybridized cells [42].

In general, when only relative proportions of different phylogenetic groups are considered, the FISH technique revealed only a very few significant changes in BCC, caused by the longitudinal transfer of the samples to the more nutrient-limited part of the reservoir (see Table 2). For instance, the significant differences in BCC between the <0.8 μm treatment (without predators) and both <5 μm and unfiltered treatments (with increased predation pressure) at both stations were observed only occasionally (see Table 4). Moreover, the robust physiological response induced by the opposite transfer (from nutrient-limited dam area to nutrient-rich inflow part) reported previously [22] did not occur.

Not surprisingly, BET became the most abundant phylogenetical group within the domain *Bacteria* after the first day of the experiment, contributing to 50–65% of the total DAPI-stained cells (Fig. 4). Similar proportions of BET were also reported by other authors [43], which suggests their general dominance in freshwater ecosystems. BET also showed the ability to outgrow the other groups of bacteria under either reduced or enhanced predation pressure (Fig. 4). Moreover, this dominance was also supported by the fastest doubling times of BET and R-BT in bacterivores-free treatments (Table 1). The proportion of the specific cluster R-BT (subgroup of BET) revealed no clear trend in different size-fractions, though it generally accounted for a large part of total bacteria in the RIVER-incubated treatments.

Proportions of the CF group, after a short initial increase, tended to decrease during the incubation period (Fig. 4). This may suggest that bacteria belonging to the CF group were more vulnerable prey for the fast developing bacterivore communities or were not efficient competitors within the whole bacterioplankton assemblage, under the rapidly changing, manipulation-induced scenario of nutrient availability. Bacteria from the CF group are usually found in nutrient-rich environments with high nutrient input and loading from the catchment area [44,45]. CF bacteria are also able to utilize the matter with a high molecular weight, such as humic compounds [46], that were present in the inflow area of the Římov reservoir.

Members of the GAM constituted the very minor part of the assemblage during the whole experiment except the last day of incubation (Fig. 4). However, a marked increase in ''vibrio-like'' bacterial morphotypes affiliated to GAM has been observed on the last day of the experiment (Fig. 6(f)). Despite the very low proportion (<5%), unproportionately to that GAM represented a very significant portion of total bacterial biomass containing five times more carbon per cell in comparison to the mean value characteristic for the rest of the assemblage at 96 h (Table 3). Although the FISH results based on the relative abundances of the broad probe-defined bacterial population showed significant differences only rarely, a morphology and bacterial biomass allocated within GAM did change markedly.

A sequential increase in the development of grazing-resistant morphotypes dominated by flocs has been observed in all grazer-exposed treatments (Fig. 5). The total flocs and filaments developed in unfiltered DAM treatment accounted for almost half of total bacterial abundance. The most filaments and floc-forming bacterial cells were detected as CF and BET members. Furthermore, we also observed filaments composed of a chain of cells (Fig. 6(d)) that hybridized with both CF319a, but also with the specific R-FL615 probe, affiliated to the *Flectobacillus* cluster (R-FL) dominating in the experiment described by [15]. The *Flectobacillus* cluster seems to profit from grazing and significantly enhanced its proportion within CF group. Thus similarly to GAM, a large shift in morphology and biomass within CF group occurred in all treatments with grazing.

The morphology of very tiny coccoid cells of the ACT group, as well as those of much larger rods affili-

ated to the R-BT cluster, remained almost unchanged across all treatments during the whole experiment. In addition, ACT and R-BT positive phylotypes were neither associated with flocs nor formed any filamentous morphotypes. ACT represented only a very small fraction (<5%) of total bacterial assemblage, but its contribution increased in the RIVER-incubated <5 μm treatment (Fig. 4). A continuous increase in this grazer-enhanced treatment could be related to the very small size and the thick cell walls of these GRAM-positive bacteria, which might be less digestible by most bacterivorous protists. Also laboratory experiment [10] indicated that this group can profit from heavy grazing pressure specifically targeting their competitors within the bacterial assemblage.

Contrary to ACT, the R-BT positive phylotypes were overrepresented when the bacterivorous predators were removed. In contrast, when the grazing pressure was enhanced in <5 μm treatments, the R-BT cluster formed smaller proportions of the assemblage, suggesting that these phylotypes with a generally high growth rate (Table 1) are also quite vulnerable to protistan predation.

Overall, our experiment documented both the non-morphological (e.g. ACT) as well as the morphological traits of grazing resistance that developed as a response to top-down manipulation (GAM, R-FL, Fig. 6) within the bacterial assemblage. The present experiment revealed marked changes in the bacterial abundance and biomass, which were tightly controlled by bacterial predators, especially HNF. The longitudinal transfer of bacterioplankton assemblage from the nutrient-rich RIVER station to the nutrient-poor DAM area of the reservoir as well as the various levels of protozoan grazing would reveal only a few significant changes in the phylogenetic composition when analyzed by means of FISH. Moreover, the data based only on changes in the relative proportions of different phylogenetic groups of bacteria targeted mostly by group-specific probes must be taken into account with a reservation. They can indicate only a negligible shift in BCC (see e.g. Table 4, Fig. 4), while a robust shift is clearly indicated in probe-detected biomass distribution within different treatments.

Furthermore, e.g., the highly specific probe, R-FL615, targeting members of the *Flectobacillus* cluster undoubtedly indicates a large shift within the CF group that has a significant impact on bacterioplankton vulnerability to protistan predation and partly also on its overall metabolism. Similarly, the large shift in size and morphology of GAM in heavily grazed treatments towards large "vibrio-like" cells suggests a compositional shift within GAM, although we could not document this shift with a FISH-probe with a finer resolution.

The growth of bacterioplankton assemblage originating from the RIVER area was comparable to that of the original environment despite the lower availability of P at the DAM area of the reservoir [47]. It is likely that

Table 4
Results of one-way ANOVA with Tukey's Multiple Comparison post test performed for pair-wise comparisons of significant differences between size-fraction treatments (<0.8 μm, <5 μm, unfiltered) at intervals of t_{24}, t_{48}, t_{72} and t_{96} incubated at the nutrient-rich area (RIVER) or nutrient-poor station (DAM)

Probe	Treatment tested	Incubation at RIVER				Incubation at DAM			
		t_{24}	t_{48}	t_{72}	t_{96}	t_{24}	t_{48}	t_{72}	t_{96}
EUB	<0.8 μm vs <5 μm	NS	NS	NS	NS	NS	NS	**<0.01**	NS
	<0.8 μm vs Unfiltered	NS	NS	NS	NS	NS	NS	NS	NS
	<5 μm vs Unfiltered	NS	NS	NS	NS	NS	NS	NS	NS
BET	<0.8 μm vs <5 μm	NS	NS	NS	NS	NS	NS	NS	**<0.01**
	<0.8 μm vs Unfiltered	NS	NS	NS	NS	NS	NS	NS	**<0.01**
	<5 μm vs Unfiltered	NS	NS	NS	NS	NS	NS	NS	NS
R-BT	<0.8 μm vs <5 μm	NS	NS	NS	NS	NS	NS	**<0.05**	NS
	<0.8 μm vs Unfiltered	**<0.05**	NS	NS	NS	NS	NS	**<0.01**	NS
	<5 μm vs Unfiltered	**<0.01**	NS	NS	NS	NS	NS	NS	NS
CF	<0.8 μm vs <5 μm	NS	NS	NS	NS	NS	NS	NS	NS
	<0.8 μm vs Unfiltered	NS	NS	NS	NS	NS	NS	NS	NS
	<5 μm vs Unfiltered	NS	NS	NS	NS	NS	NS	NS	NS
GAM	<0.8 μm vs <5 μm	NS	NS	**<0.05**	NS	NS	NS	**<0.05**	NS
	<0.8 μm vs Unfiltered	NS	NS	**<0.05**	NS	NS	NS	**<0.05**	NS
	<5 μm vs Unfiltered	NS	NS	NS	NS	NS	NS	NS	NS
ACT	<0.8 μm vs < 5 μm	NS	NS	NS	**<0.01**	NS	NS	NS	NS
	<0.8 μm vs Unfiltered	NS	NS	NS	NS	NS	NS	NS	NS
	<5 μm vs Unfiltered	NS	NS	NS	**<0.01**	NS	NS	NS	NS

Tested were changes in the relative proportions (as % of total DAPI-stained bacterial cells) of six different probe-defined phylogenetic groups of bacteria affiliated to the groups of EUB, BET, R-BT, CF, GAM and ACT (see Fig. 4). The significant differences are shown in bold (**$p < 0.05$**, **$p < 0.01$**). NS – not significant.

the relative proportions of different phylogenetic groups of bacteria were mainly controlled by bottom-up factors at both stations. On the other hand, top-down factors induced marked changes in morphology and biomass within selected probe-defined bacterial groups. In comparison to protists, the effects of viruses on bacteria were negligible.

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