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Shifts in bacterial community composition associated with different microzooplankton size fractions in a eutrophic reservoir

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Abstract

Using oligonucleotide probes for *Eubacteria* and four eubacterial subgroups, we monitored changes in bacterial community composition (BCC) with different degrees of grazing pressure, as follows: unfiltered water (UNF, all bacterivores present); <20 μm (bacteria, heterotrophic nanoflagellates [HNF], and small ciliates); <5 μm (bacteria and HNF only); and <1 μm or <0.8 μm (bacteria only), incubated in dialysis bags. Experiments were conducted in the Římov Reservoir (South Bohemia) during the clear-water phase (experiment I), a period of low protistan grazing pressure on reservoir bacterioplankton, and during the late summer phytoplankton peak (experiment II), a period of high protistan grazing pressure. In both experiments, there was a significant shift in BCC in the <5 μm treatments, which came in the form of increased proportions of alpha subclass of the class Proteobacteria (ALF) and Cytophaga/Flavobacterium group (C/F), corresponding with increased bacterivory of the ungrazed HNF populations. Changes in BCC in other experimental treatments were related to preincubation protistan grazing pressure. In experiment I, bacterioplankton were subjected to negligible protistan bacterivory in the reservoir and did not show a change in BCC in the predator-free treatment (<1 μm), while BCC changed in treatments that yielded increases in protistan bacterivory compared with that in the ambient reservoir water. In experiment II, a significant shift in BCC was induced when bacterioplankton that were subjected to heavy predation pressure in the reservoir were transferred into the predator-free treatment. Treatments that induced small changes in protistan grazing pressure (<20 μm and UNF in experiment II) did not show significant BCC shifts. Proportions of filamentous bacteria (>4 μm) increased in treatments that yielded large increases in bacterivory. Filament formation also showed season-specific features; in experiment I, all filaments belonged into ALF, whereas in experiment II, most of them hybridized with the C/F probe. We conclude that the sudden shifts that violate the established balance between bacterial production and the protist-induced bacterial mortality led to the significant shifts in cell morphology and BCC.

Remarkable changes in bacterioplankton abundance, morphologic diversity, and activity have been associated with protistan bacterivory (e.g., Sanders et al. 1992; Jürgens and Güde 1994). However, very little is known about how morphologic and physiologic changes are related to changes in the relative abundances of bacterial genotypes that define bacterial community composition (BCC). Progress has been limited because of the lack of methods available to describe BCC in natural aquatic systems.

In the past few years, new information on BCC in aquatic

environments has been obtained using *in situ* hybridization with fluorescently labeled oligonucleotide ribosomal ribonucleic acid (rRNA)-targeted probes (Amann et al. 1990). Initially, this method was used in freshwater environments that had large, active bacteria with a high number of ribosomes (e.g., activated sludge; Wagner et al. 1993). Recently, distinct differences in BCC have been shown among natural systems (e.g., Methé et al. 1998) as well as within a given system, among seasonal phases (Alfreider et al. 1996; Pernthaler et al. 1997a). Similarly, bacteria living on limnetic organic aggregates have distinct BCCs (Weiss et al. 1996).

Factors that induce changes in BCC have become one of the central issues of aquatic microbial ecology. The highly flexible relationship between bacteria and the bacterivorous protists offers an almost ideal model for studies that involve the responses of bacterial communities to changing grazer control. Some of the more general features of this relationship are already known. Protozoan grazing on bacteria is size selective (Chrzanowski and Šimek 1990; González et al. 1990b), and thus, it induces a strong morphologic shift in bacterial communities toward cells that are resistant to graz-

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ing (Sibbald and Albright 1988; Bianchi 1989; Güde 1989). Different bacterial strains of similar morphology can be consumed and digested with different rate and growth efficiencies (Mitchell et al. 1988; González et al. 1990a; Irriberri et al. 1994). Finally, through numerous feedback mechanisms, grazing is known to affect substrate availability for bacteria, resulting in a tight relationship between protistan grazing and the metabolic and physiologic activities of bacterio-plankton (e.g., Šimek et al. 1990; Sanders et al. 1992).

Until quite recently, it was unclear whether morphologic and physiologic changes could reflect phenotypic plasticity of strains present in bacterial communities or whether changes corresponded with shifts in BCC. In a laboratory chemostat study of a complex bacterial assemblage growing on algal exudates, we found that a selective flagellate bacterivore, present in high abundances (10^4 – 10^5 ml⁻¹), had a strong impact on bacterial morphology, viability, and BCC (Šimek et al. 1997). The flagellate also induced contrasting survival strategies among bacterial strains (Pernthaler et al. 1997b), with shifts to high division rates of some heavily grazed genotypes, whereas in others, grazing resistance was mediated through an increase in cell size. Other laboratory studies, conducted on a bacterial community that consisted of only two species, found species-specific formation of grazer-resistant filaments (Hahn and Höfle 1998).

While the laboratory evidence is compelling, there is as yet only preliminary field-study-derived data on changes in BCC that can be directly linked to bacterial consumers (Šimek et al. 1998). It is unknown whether protistan bacterivory under typical in situ concentrations of bacteria and protists in freshwaters is indeed one of the driving forces that shapes BCC of natural bacterial communities. In order to examine this, in two experiments we determined the impact of different plankton size fractions (tentatively analyzed by using in situ hybridization with oligonucleotide rRNA-targeted probes) on BCC.

Methods

Study site and experimental design—Experiments were conducted in the mesoeutrophic Římov Reservoir (South Bohemia; for details see Šimek et al. 1995). The sampling site was located above the former river valley (a 30-m depth), about 250 m from the reservoir dam. Water samples were taken with a 2-liter Friedinger sampler from a depth of 0.5 m (10 samples), and a final volume of 20 liters was mixed in a 50-liter plastic container. Five liters of the mixed sample were used for further microzooplankton and zooplankton analyses.

Experiment I was run during the late clear-water phase (9–13 June 1997; water temperature, 18–19°C; and chlorophyll *a* concentration, 5 mg L⁻¹). Water samples were sequentially size-fractionated through 20- μ m nylon mesh and then through 5- and 1- μ m pore-size Poretics filters (47-mm diameter) into the following fractions: <20 μ m (bacteria, heterotrophic nanoflagellates [HNF], and small ciliates); <5 μ m (bacteria and HNF only); and <1 μ m (bacteria only) (compared with unfiltered samples in which all bacterivores were present); these fractions will be designated throughout

the text as <20 μ m, <5 μ m, <1 μ m, and unfiltered water (UNF) treatments, respectively. The last 1- μ m filtration step was conducted in sterilized glass Poretics holders in order to minimize HNF contamination. The water fractions were placed in 2l pretreated (deionized water-rinsed and -boiled) dialysis tubes (diameter, 75 mm; molecular weight cut-off 12,000–16,000 daltons; Poly Labo). The dialysis bags were incubated in the reservoir at a depth of 0.5 m, oriented horizontally in Plexiglas holders. Subsamples (~250 ml) were taken at times 0, 48, and 96 h. The microbiological parameters of the water surrounding the bags were analyzed at times 0 and 96 h.

Experiment II was conducted during the late phase of a summer phytoplankton peak (12–18 September 1997; water temperature, 17–19°C; and Chl *a* concentration, 17 mg L⁻¹). Basically we used an almost identical experimental design as that described for experiment I, but all the experimental treatments were replicated, the incubation of dialysis bags was prolonged, and subsamples taken at times 0, 72, and 144 h. One of the replicated <1 μ m treatments was contaminated by small HNF in experiment I (likely during the sample filtration) and had to be omitted from the data processing. Thus, in order to minimize the possibility of the HNF contamination of the predator-free treatments in experiment II, instead of <1 μ m treatment, we filtered the sample through 0.8- μ m pore-size filters (Poretics). An additional treatment was added; to bags of <5 μ m filtered water, laboratory-grown populations of HNF were added, as described below.

A day before the experiment began, 3.5 liters of water from the study site was filtered through 5- μ m pore-size Poretics filters (47- μ m diameter) using low vacuum. Filtered water (2.8 liters) was further filtered through 2- μ m pore-size filters to remove auto- and heterotrophs of >2 μ m. Then, bacteria from ~2.5 liters of the 2- μ m filtrate were concentrated onto 0.2- μ m pore-size Poretics filters (47-mm diameter) in 200-ml portions. The filters were briefly sonicated in ~400 ml of the 0.2- μ m filtrate, which resulted in detachment of bacteria from the filter surface. This procedure yielded a solution with about 20×10^6 bacterial cells ml⁻¹ (i.e., ~six times more concentrated than the natural bacterio-plankton concentration at the beginning of experiment II). The 400 ml of bacteria-enriched water inoculated with 200 ml of the 5- μ m filtrate (containing HNF) was incubated for 24 h; at the end of the incubation period, ~ 18×10^3 HNF ml⁻¹ was yielded. Bags enriched with HNF (assigned as <5 μ m + HNF treatment) received the equivalent of 300 ml of the HNF culture, which had been washed free of bacteria using rinses over 1- μ m filters (for details of this procedure, see Šimek et al. 1997). The additions resulted in a significantly increased HNF abundance at the beginning of the experiment compared with that in the unenriched, <5 μ m treatments.

Bacterial abundance and biomass—Subsamples were fixed with formalin (2% final concentration), stained with 4', 6-diamino-2-phenylindole (DAPI, final concentration 0.2% weight/volume), and enumerated using epifluorescence microscopy (Olympus BX-60). Between 400 and 600 bacteria were sized by semiautomatic image analysis (Lucia; Laboratory Imaging). For more details, see Posch et al. (1997).

As cell width showed very little variability, the cell length was the most important factor in determining cell volume. The cells that were longer than 4 μm were assumed to be protected against grazing by most of the bacterivorous HNF (Šimek et al. 1997) and ciliates. Correspondingly, we used this criterion to split up image analysis data into the small-celled ($<4 \mu\text{m}$) bacterial biomass that is edible for protists, in comparison with the grazing-resistant bacterial volume biomass of filamentous cells of $>4 \mu\text{m}$.

Bacterial production—Bacterial production (experiment II only) was measured via thymidine incorporation, as modified from Riemann and Søndergaard (1986). Duplicate 5-ml subsamples were incubated for 30 min at in situ temperature with 10 nmol L⁻¹ of [methyl-³H] thymidine (Amersham) and were then preserved with neutral-buffered formalin (2% final concentration), filtered through 0.2- μm membrane filters (Poretics), and extracted 10 times using 1 ml of ice-cold 5% trichloroacetic acid (for details, see Šimek et al. 1995). Replicate blanks prefixed with 2% formalin were processed in parallel. An empirical conversion factor (ECF) between thymidine incorporation rate and bacterial cell production rate was determined using data from the replicated $<0.8 \mu\text{m}$ treatments. The cell production rate was calculated from the slope of the increase of \ln bacterial abundance over time (0, 24, and 72 h). Our determined ECF ($2 \pm 0.58 \times 10^{18}$ cells mol⁻¹ thymidine) was used for calculations.

Fluorescent in situ hybridization with group-specific oligonucleotide rRNA-targeted probes—Analysis of BCC was carried out by in situ hybridization with fluorescence oligonucleotide probes on membrane filters (Alfreider et al. 1996; Glöckner et al. 1996). For details of the hybridization procedure and error estimates, see Pernthaler et al. (1997b). Briefly, bacterial cells from 10–20-ml subsamples were concentrated on white, 0.2- μm pore-size filters (Poretics; 47-mm diameter) and were then fixed on membrane filters by overlaying the filters with 4% paraformaldehyde in phosphate-buffered saline (pH 7.2) and stored at -20°C (Alfreider et al. 1996). The five different oligonucleotide probes (MWG Biotech) were targeted to the kingdom Bacteria (*Eubacteria*, EUB); to the alpha, beta, and gamma subclasses of the class Proteobacteria (ALF, BET, and GAM), and to the Cytophaga/Flavobacterium group (C/F) (Amann et al. 1995). The probes were fluorescently labeled with the indocarbocyanine dye CY3 (BDS). After hybridization, filter sections were stained with DAPI, and the percentage of hybridized bacterial cells was enumerated by epifluorescence microscopy.

Protozoan grazing and abundance—Protozoan grazing on bacterioplankton was estimated using fluorescently labeled bacterioplankton (FLB; Sherr and Sherr 1993) concentrated from the reservoir water following the method of Šimek and Straškrabová (1992). FLB uptake experiments were run in each of the bags containing protists at each time point as well as in untreated reservoir water at the beginning and end of both experiments. Samples (150 ml) were dispensed into acid-soaked and -rinsed 250-ml flasks and were preincubated at in situ temperature for 15 min (in order to reduce handling shock). Flagellate and ciliate uptake rates were determined

in the same treatment in which FLB were added at 5–15% of bacterial natural abundances. In order to conduct protozoan enumeration and tracer ingestion determinations, 25-ml subsamples were taken at 0, 5, 10, 15, 20, and 30 min after tracer addition and were fixed by adding 0.5% of alkaline Lugol's solution, immediately followed by 2% borate-buffered formalin (final concentration) and several drops of 3% sodium thiosulfate (in order to clear the Lugol's color) (Sherr and Sherr 1993).

We determined ciliate grazing rates in time series from 5–15-min subsamples and flagellate grazing rates in subsamples from 10–30 min, respectively. Five-milliliter (flagellate) or 20-ml (ciliate) subsamples were stained with DAPI, filtered through 1- μm black Poretics filters, and inspected via epifluorescence microscopy. Nonpigmented HNF and plastic flagellates were differentiated. At least 40 ciliates and 50 flagellates were inspected for FLB ingestion in each sample. In order to estimate total protozoan grazing, we multiplied average uptake rates of ciliates and flagellates by their in situ abundances. For ciliate identification, samples were fixed with Bouin's fixative (5%). Ten to forty milliliters of a sample were filtered onto a nitrocellulose membrane filter (1.2- μm pore size; Millipore), mounted in agar, fixed with formalin, and protargol stained at a temperature of 40–60°C, according to the method of Skibbe (1994). Ciliates were identified based on Foissner and Berger (1996) and on references therein.

Zooplankton—At the beginning of the experiments, zooplankton of $>100 \mu\text{m}$ were concentrated from 5 liters (see above), preserved in 4% formalin (final concentration), and quantified by direct microscopic counting of several subsamples (McCauley 1984). At the end of the experiments, the water remaining in the dialysis bags containing unfiltered samples (~600–800 ml) was concentrated, and zooplankton composition was tentatively analyzed. Rotifers were enumerated separately from unscreened water as follows: samples (0.5–1 liter) were preserved with acid Lugol's solution (1% final concentration) and after sedimentation were post-fixed with Bouin's fixative (5% final concentration), which was added into the 10-fold concentrated samples. The concentrated subsamples (5 ml) were filtered onto nitrocellulose membrane filters (Synpor 1, 4- μm pore size, 10-mm diameter; Barvy Laky) and stained with carbolerythrosine (Sorokin and Overbeck 1972). The whole filter area was inspected.

Chl a—Chl *a* concentrations in prescreened water ($<100 \mu\text{m}$) were determined after filtration through Whatman GF/C filters. Filters with retained seston were ground, extracted in 90% acetone, and measured spectrophotometrically after the method of Lorenzen (1967).

Statistical analysis—In order to test whether changes in BCC occurring within a respective treatment (dialysis bag, size fraction) during the experiment were statistically significant, a chi-square test was applied. For each treatment, proportions of detected cells that had been hybridized with each rRNA-targeted probe were entered into a contingency table that contained four columns, which corresponded to

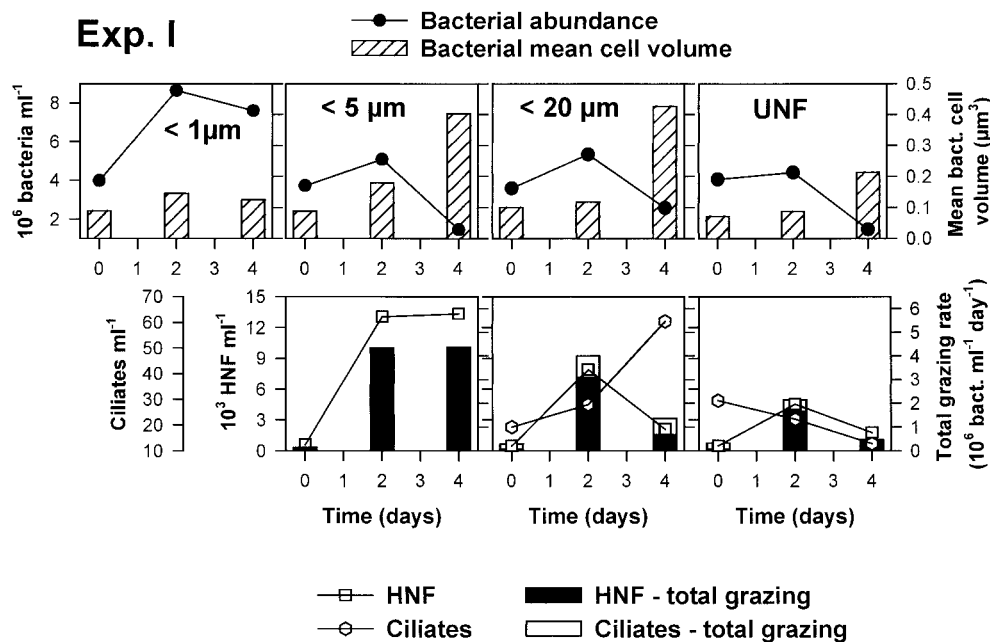


Fig. 1. Results of experiment I, changes in microbial parameters in different size-fractionation treatments ($<1 \mu\text{m}$, $<5 \mu\text{m}$, $<20 \mu\text{m}$, and unfiltered reservoir water [UNF]) exposed in dialysis bags. Upper panels: bacterial abundances and mean cell volumes; lower panels: abundances of heterotrophic nanoflagellates (HNF), ciliates, and total protistan bacterivory subdivided into HNF and ciliate grazing.

the probes used (ALF, BET, GAM, and C/F), and three rows, which corresponded to sampling days (0, 2, 4 or 0, 3, 6 in experiments I and II, respectively). Row-to-row variability was tested. The analyzed bacterial cells were considered to be randomly selected subsamples of the bacterial populations inside the bags. As numbers of DAPI-stained cells varied (400–600 cells counted per sample), counts of hybridized cells were normalized to 400 DAPI-stained cells. In experiment II, average proportions from the two replicates were examined with the chi-square test. Using these tests, differences occurring within a respective treatment could be tested, but different treatments could not be compared. However, the presence of highly significant changes observed consistently in both experiments within certain treatments (e.g., $<5 \mu\text{m}$ and $<20 \mu\text{m}$) contrasted with the absence of significant changes in untreated (unfiltered) or predator-free variants indicated that the treatments did affect BCC.

Results

Experiment I: Clear water populations—Bacterial numbers in the bag without protistan predators ($<1 \mu\text{m}$) sharply increased during the first 2 d of the incubation, then they slightly decreased (Fig. 1). In the other treatments ($<5 \mu\text{m}$, $<20 \mu\text{m}$, and UNF), there was a smaller initial increase of bacteria followed by a marked decrease during the second half of the experiment. The drop in bacterial abundance was paralleled by increases in number and activity of bacterivores, especially in HNF during the first 2 d of incubation. During the second half of the experiment, changes in HNF abundance corresponded with the absence or presence of

higher trophic levels in bags (Fig. 1). HNF in the $<5 \mu\text{m}$ treatment reached a high abundance, around 14×10^3 cells ml^{-1} , whereas in the $<20 \mu\text{m}$ and UNF treatments, HNF dropped between the second and fourth days of the incubation, probably because of predation by abundant ciliates in the $<20 \mu\text{m}$ bag or by ciliates and metazooplankton in the UNF treatment.

Ciliate dynamics varied with the experimental treatment: in the $<20 \mu\text{m}$ water, they increased to 61 cells ml^{-1} , and they became as important bacterivores as HNF (Fig. 1), whereas in the UNF treatment, ciliates were seemingly suppressed by a competition with or by the grazing activity of metazooplankton. Among ciliates present in the $<20 \mu\text{m}$ and UNF treatments, the bacterivorous or omnivorous species *Halteria grandinella* and *Pelagohalteria viridis* dominated both numerically and in terms of their status as bacterivores. The most important groups of zooplankton that were potentially preying upon protists in the UNF treatment (Table 1) were coarse, filter-feeding cladocerans—*Daphnia galeata*, *Bosmina longirostris*, copepods, and several filter-feeding rotifers and raptorial *Asplanchna* sp.

Marked changes occurred in bacterial mean cell volumes, proportions of filamentous bacteria, and BCC (Figs. 1, 2). The changes were associated with abundances and activities of bacterivores present in the experimental treatments. Increases in protistan bacterivory, especially in the $<5 \mu\text{m}$ and $<20 \mu\text{m}$ treatments, corresponded with (1) increases in mean cell volume and the appearance of large, filamentous bacteria, all of the alpha subgroup of Proteobacteria (ALF), accounting for 5–7% of the total DAPI-stained cells; (2) a shift of about 30–46% of total bacterial biomass into gra-

Table 1. A comparison of zooplankton composition at the beginning of the experiments in the reservoir water and at the end of the experiments in the unfiltered treatments. Only those species or groups of zooplankton, the abundance of which was >1 individual per liter, are listed.

Zooplankton composition	Experiment I		Experiment II	
	Reservoir water, day 0	Unfiltered treatment, day 4	Reservoir water, day 0	Unfiltered treatment, day 6
	(individuals liter ⁻¹)		(individuals liter ⁻¹)	
<i>Daphnia galeata</i>	23	11	17	14
<i>Bosmina longirostris</i>	35	18	3	5
<i>Eubosmina coregoni</i>	4	3	3	0
<i>Diaphanosoma brachyurum</i>	3	2	27	25
<i>Ceriodaphnia quadrangula</i>	1	2	114	55
<i>Eudiaptomus gracilis</i> , adults	9	6	6	5
<i>Eudiaptomus gracilis</i> , copepodites	7	3	4	6
Adults of cyclops*	37	19	14	9
Copepodites of cyclops	35	25	6	12
Nauplii of all Copepoda	40	52	60	35
Filter-feeding rotifers†	940	380	740	620
<i>Asplanchna</i> sp.	460	420	120	200

* Adults of *Cyclops vicinus*, *Mesocyclops leukarti*, *Thermocyclops* sp., and *Acanthocyclops* sp.

† *Keratella cochlearis*, *K. quadrata*, *Conochilus* sp., *Filinia* sp., and *Brachionus* sp.

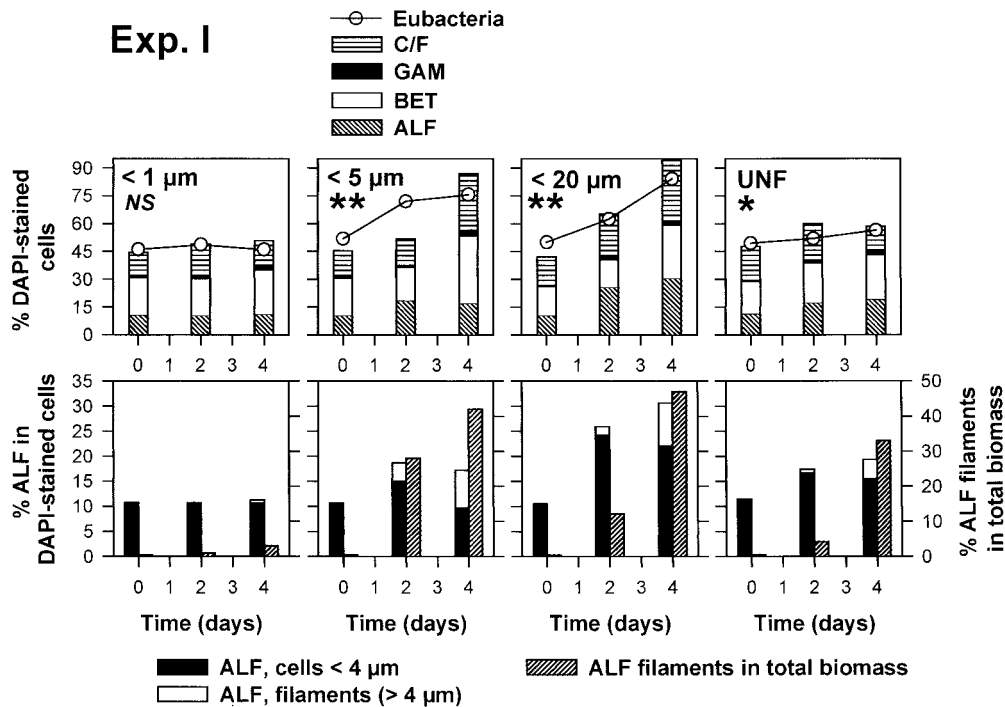


Fig. 2. Results of experiment I. Upper panels: proportions of ALF, BET, and GAM subclasses of the class Proteobacteria, Cytophaga/Flavobacterium group (C/F), and of *Eubacteria* in total DAPI-stained bacterial cells. Lower panels: a detailed view of the proportions of cells (<4 μm and >4 μm) (filaments) within ALF and the proportions of the filamentous ALF in total bacterial biomass. Note that all the filaments hybridized with the ALF probe. For additional explanation, see caption for Fig. 1. * $P < 0.05$; ** $P < 0.01$, significance level of differences in proportions of the subgroups detected within the treatments among days 0, 2, and 4, as tested with a chi-square test. NS, not significant.

Table 2. Percentage of bacterial standing stock daily removed by protistan grazing in all the experimental treatments compared to the ambient reservoir water.

Experimental day	< 5 μm			Unfiltered	Reservoir
	< 5 μm	+ HNF	< 20 μm		
Experiment I					
0	4	—	8	8	8
2	85	—	75	48	ND
4	301	—	53	31	5
Experiment II					
0	19	69	28	31	31
3	264	118	128	74	ND
6	75	53	50	42	41

* HNF, heterotrophic nanoflagellates; ND, not determined

ing-resistant thin ALF filaments ($>4 \mu\text{m}$; see Fig. 2); and (3) a significant BCC shift in the proportions of subgroups of *Eubacteria* between the beginning and end of the experiment (chi-square = 20.2; df = 6; $P < 0.002$ in the $<5 \mu\text{m}$ treatment and chi-square = 16.7; df = 6; $P < 0.01$ in the $<20 \mu\text{m}$ treatment). The shifts consisted mainly of higher proportions of the beta subgroup of Proteobacteria (BET) and C/F group and, to a lesser degree, of shifts in the proportion of ALF (Fig. 2). Only the gamma subgroup of Proteobacteria (GAM) showed no clear changes. However, as it accounted only for 1–3.5% of total DAPI-stained bacteria, it was of minor importance.

Another conspicuous phenomenon, possibly related to high protistan grazing pressure in the $<5 \mu\text{m}$ and $<20 \mu\text{m}$ treatments, was an increase in the DAPI-stained bacteria that hybridized with the probe for *Eubacteria* (from ~ 50 to 75 and 85%, respectively; Fig. 2). In comparing bacterial abundance and total protistan grazing rate (Fig. 1) at the beginning of experiment I in the UNF treatment, the total protistan grazing would remove only $\sim 8\%$ of bacterial standing crop daily, but this percentage increased to 85 and 301% in the $<5 \mu\text{m}$ treatment and to 75 and 53% in the $<20 \mu\text{m}$ treatment on the second and fourth experimental days, respectively (Table 2).

A small increase in the ALF filaments and less marked BCC changes (chi-square = 12.8; df = 6; $P < 0.047$) were also observed in the UNF treatment. In contrast, samples taken from the reservoir at the beginning and end of the experiment (data not shown) did not show significant differences in BCC (chi-square = 5.9; df = 3; $P < 0.091$). However, in the UNF treatment, protozoan abundance and bacterivory reached much higher values than in reservoir water. Thus, at the end of the experiment, there were 1.79×10^3 HNF ml^{-1} in the UNF treatment (Fig. 1) but only 0.3×10^3 HNF ml^{-1} in the reservoir water (data not shown), resulting in ~ 6 times stronger grazing impact of protists in this treatment compared with the ambient reservoir water (Table 2). In the $<1 \mu\text{m}$ treatment, no remarkable changes were found in the bacterial mean cell volume (Fig. 1), and no significant shift in BCC (Fig. 2) was observed (chi-square = 5.67; df = 6; $P < 0.46$).

Experiment II: Post-summer bloom populations—In experiment II, conducted during the late phase of a summer

phytoplankton peak, changes in bacterial abundance in the $<0.8 \mu\text{m}$ treatment showed a pattern that was quite similar to that found in experiment I in the corresponding treatment (i.e., $<1 \mu\text{m}$; Figs. 1, 3). In contrast, we did not find any clear trend in changes of bacterial abundance in all other treatments, but we did note an increase in mean bacterial cell volume, especially during the first 3 d of the incubation. The largest shift, from ~ 0.1 to $0.42 \mu\text{m}^3$, concerned bacteria that had been subjected to a sharp increase in grazing pressure—the treatment in which HNF were added ($<5 \mu\text{m} + \text{HNF}$). In this treatment, at the very beginning of the experiment, grazing-induced bacterial mortality exceeded bacterial production (Fig. 3).

HNF abundances showed very similar trends in all the HNF-containing treatments; they increased sharply at the beginning and dropped during the second part of the experiment (surprisingly, this pattern also followed in the treatments that did not contain other predators [$<5 \mu\text{m}$ and $<5 \mu\text{m} + \text{HNF}$]). However, HNF decreases corresponded with large shifts in average bacterial cell volume in those two treatments (Fig. 3). The decrease in HNF in the <20 and UNF treatments corresponded with increases in ciliates, which suggested a grazer control of HNF by a steeply increasing population of ciliates in the $<20 \mu\text{m}$ treatment (to $126 \text{ cells ml}^{-1}$) and a combined grazing control by ciliates and metazooplankton in the UNF treatment. The metazooplankton composition had several features that were different from those characterizing metazooplankton in experiment I (Table 1): the small, fine, filter-feeding cladocerans *Diaphanosoma brachyurum* and *Ceriodaphnia quadrangula* numerically dominated among larger zooplankters; *Bosmina longirostris*, copepods, and the raptorial rotifer *Asplanchna* sp. were less abundant in experiment II.

As in experiment I, conspicuous changes occurred in bacterial size, morphology, and BCC in the different experimental treatments (Figs. 3, 4). In order to examine the possibility that our experimental treatment (the size fractionation or HNF enrichment) introduced some shift in BCC from that of the original reservoir population, we tested the differences prior to and after the treatments. At time zero, no significant differences in BCC were found among the experimental treatments (chi-square = 17.3; df = 12; $P < 0.16$).

In contrast to experiment I, during a phase of negligible protistan bacterivory in the reservoir, in experiment II the reservoir bacterioplankton had experienced strong protistan predation. The transfer from the grazing-exposed reservoir water (31% of bacterial standing stock removed daily by protists; Table 2) into the dialysis bags without bacterivores ($<0.8 \mu\text{m}$) yielded an increase in the mean cell volume and a significant change in BCC between the beginning and end of the experiment (chi-square = 17.9; df = 6; $P < 0.007$). The change was a large increase in the proportion of BET and, to a lesser extent, in the proportion of filamentous bacteria, all of which hybridized with the EUB probe and only a small part of which hybridized with the more specific C/F probe (see Fig. 4). When we checked for the possible presence of HNF in this supposedly ungrazed $<0.8 \mu\text{m}$ treatment, no HNF were found in both replicates after 3 d, but by the end of the experiment, we found <50 HNF ml^{-1}

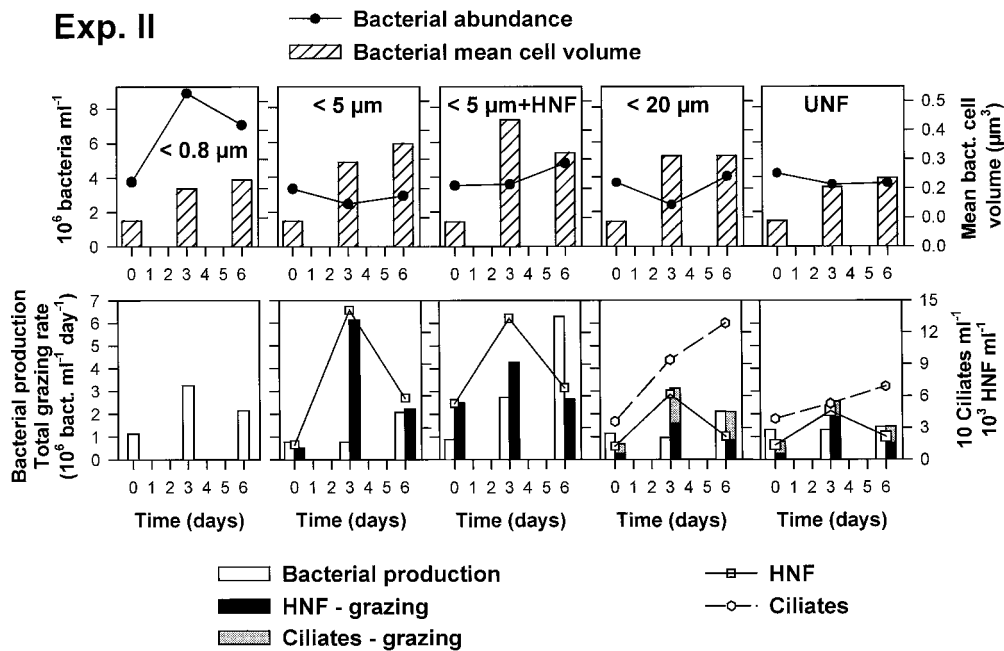


Fig. 3. Results of experiment II, changes in microbial parameters in different size-fractionation treatments (<1 μm , <5 μm , <5 μm + heterotrophic nanoflagellates [HNF], <20 μm , and unfiltered reservoir water [UNF]) exposed in dialysis bags. Upper panels: bacterial abundances and mean cell volumes; lower panels: abundances of HNF, ciliates, bacterial production, and total protistan bacterivory (both in 10^6 bacteria ml^{-1} d^{-1}) subdivided into HNF and ciliate grazing. The data are mean values of the duplicate treatments.

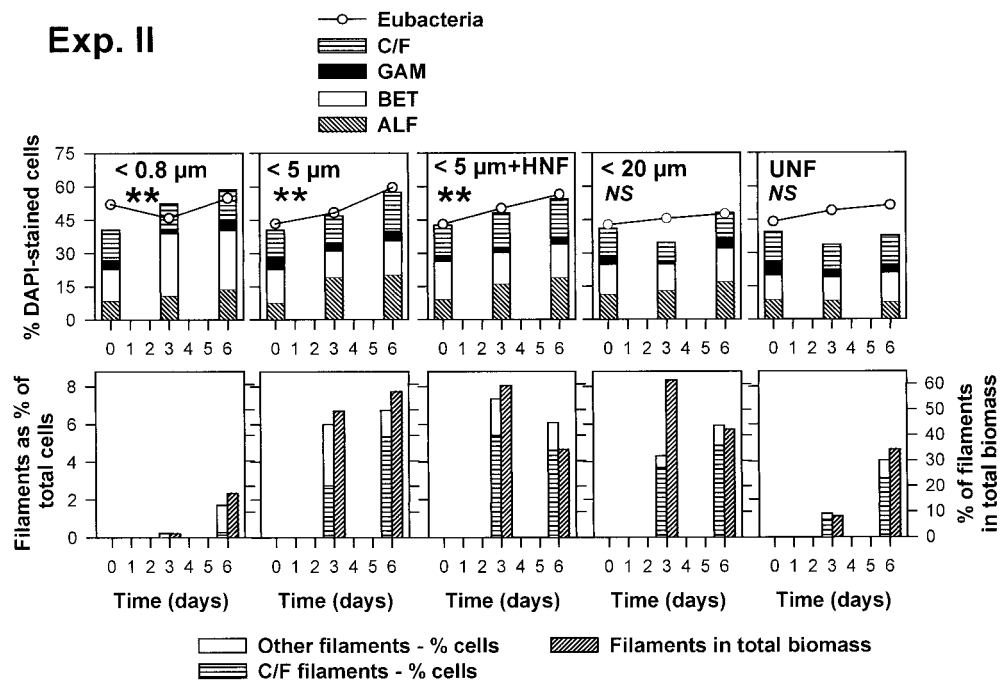


Fig. 4. Results of experiment II. Upper panels: proportions of bacteria belonging to ALF, BET, GAM, and C/F subgroups and of Eubacteria in total DAPI-stained bacterial cells. Lower panels: proportions of filaments (>4 μm) hybridized with the C/F probe and proportions of other filamentous cells in total DAPI-stained bacteria and proportions of all the filamentous bacteria in total bacterial biomass. Differences between the treatments were tested on days 0, 3, and 6 with a chi-square test. The data are mean values of the duplicate treatments. For further explanation, see captions for Figs. 2 and 3.

in one of them (data not shown). Considering the cell-specific grazing rate of HNF on bacteria in all the “grazed treatments” (~ 15 bacteria cell⁻¹ h⁻¹, data not shown) and the bacterial abundance and production in the $<0.8 \mu\text{m}$ treatments between the third and sixth days of the experiment (Fig. 3), this very low number of HNF should not have affected bacterial dynamics.

There were many commonalities in the $<5 \mu\text{m}$ and $<5 \mu\text{m} + \text{HNF}$ treatments. First, HNF abundance and grazing peaked in the middle of the experiment, which resulted in a strong excess of bacterial grazing mortality compared to the numbers of newly produced bacterial cells (Fig. 3). In both treatments, there was a trend toward an increasing proportion of DAPI-stained cells hybridized with the EUB probe (from 45 to $\sim 60\%$; Fig. 4). We found significant changes in BCC (chi-square = 16.9 and 17.0; df = 6; $P < 0.01$ for the $<5 \mu\text{m}$ and $<5 \mu\text{m} + \text{HNF}$ treatments, respectively), which consisted of a sharp increase in the proportion of ALF and a marked rise in the proportion of filamentous bacteria (6–7% of the total DAPI-stained bacteria), all of which hybridized with the EUB probe, with most of them belonging to the C/F group (Fig. 4). Finally, in both treatments, there was a morphologic shift—at the end of the experiment, 40 to 59% of the bacterial biomass consisted of HNF grazing-resistant cells of $>4 \mu\text{m}$.

The $<20 \mu\text{m}$ treatment represented an intermediate degree of bacterivory between the “heavily grazed treatments,” with HNF as the sole bacterivores ($<5 \mu\text{m}$ treatments), and the experimental treatment, where $>50\%$ of bacterivory was the result of ciliates (Fig. 3). The ciliate community shifted during the course of the study; initially, it consisted of a high proportion of small oligotrichs (*Halteria grandinella* and small strobiliids, data not shown), but then its composition shifted toward one that was dominated by *Coleps* sp. and scuticociliates, especially *Cinetochilium margaritaceum*, and a *Cyclidium* sp. Thus, in parallel with a steep ciliate abundance increase, there was a drop in HNF abundance as well as a drop in total protistan and ciliate bacterivory. On the other hand, the heavily grazed-upon bacterial populations were similar to those found in other treatments (i.e., a high proportion of filamentous bacteria mostly belonging to C/F and a general trend of the increase in the proportion of ALF within *Eubacteria* [Fig. 4]). Although we observed a slight shift in BCC, it was not significant (chi-square = 9.98; df = 6; $P < 0.13$).

The UNF treatment yielded the smallest differences in experiment II. Ciliate numbers only slightly increased (Fig. 3), and protistan bacterivory was comparable to that found in the ambient reservoir water. The proportion of bacterial production or standing stock removed daily by protists was quite similar at the start and end of the study in the UNF treatment compared with those in the reservoir water (Fig. 3, Table 2). Nevertheless, by the end of the study, we did observe a certain increase in the proportion of filamentous, predominantly C/F bacteria (Fig. 4); however, there was no overall significant BCC shift (chi-square = 5.24; df = 6; $P < 0.51$). Comparing BCC at the beginning and end of experiment II in the UNF treatment (Fig. 4) and reservoir water (data not shown), we verified that during the course of the

study, there was no significant deviation from the BCC found in the reservoir (chi-square = 8.42; df = 6; $P < 0.21$).

Discussion

Although laboratory studies have suggested that protistan bacterivory could be responsible for sudden shifts in BCC in aquatic ecosystems (Šimek et al. 1997; Hahn and Höfle 1998), to date only preliminary evidence has been presented to indicate that protistan bacterivory could influence BCC at natural concentrations of bacteria and protists (Šimek et al. 1998). In order to examine the role of protists, our approach was a compromise between typical laboratory studies that use high concentrations of simple predator and prey mixtures and a detailed field study, in which in-depth description of temporal changes in a microbial community could yield data that are difficult to analyze. We chose to examine a small number of parameters in natural communities subjected to simple manipulation via size fractionation and incubation in dialysis bags (which were exposed directly in the reservoir). We did not intend to experimentally modify the substrate supply for bacterioplankton; however, we did anticipate that there might be differences induced by changing the availability and recycling of substrate and nutrients, caused by modifying protozoan bacterivory in experimental treatments. By using dialysis bags, we assumed that qualitative and quantitative differences in substrate supply (between ambient reservoir water and the experimental treatments) had been minimized. Our methods allowed us to characterize most of the bacterioplankton. By using the rRNA-targeted oligonucleotide probe for *Eubacteria*, we identified at least 45–65% of the total DAPI-stained bacterial cells, a range of values similar to those reported in oligo- and mesotrophic lakes (Alfreider et al. 1996; Glöckner et al. 1996; Pernthaler et al. 1997a). However, the proportion of cells visualized with the EUB probe reached $>85\%$ in the heavily grazed treatments (Fig. 2). This may indicate the enhanced physiological state (i.e., a higher number of ribosomes in bacterial cells) of the heavily grazed-upon segment of the bacterial community. This enabled us to successfully target a higher proportion of *Eubacteria* in the system, whereas slowly growing cells, which presumably contain low numbers of ribosomes and are thus undetectable, could have been removed or could have become numerically less important. This phenomenon has previously been reported in chemostat studies (Šimek et al. 1997). In general, a sum of ALF, BET, GAM, and C/F hybridized cells matched well with the proportion of EUB-detectable cells in the total bacterial numbers (see Figs. 2, 4). It is worth noting that our data on the lack of changes in BCC in treatments in which bacteria were not subject to a large change in grazing pressure support the notion that simple containment or size fractionation does not inevitably lead to large shifts in the composition of bacterioplankton.

We conducted our studies during two contrasting periods with regard to the role of protists in the reservoir bacterivory: the clear-water phase and late summer phytoplankton peak (Figs. 1, 3, Table 2). The BCC shift found in both experiments with no top-down control of HNF ($<5 \mu\text{m}$ and $<5 \mu\text{m} + \text{HNF}$ treatments) can be attributed to a heavy and

selective grazing impact of HNF. No comparable shift was detected in either ambient reservoir water or in the flagellate-free <1 and $<0.8 \mu\text{m}$ treatments (Figs. 2, 4). The only difference between the HNF-enriched and -unenriched treatments was the initial rate of bacterivory, which accelerated the imbalance between bacterial growth and mortality rates. In experiment II, by the third day this resulted in almost 60% of the biomass of filamentous ($>4 \mu\text{m}$) for HNF grazing-resistant bacteria in the $<5 \mu\text{m} + \text{HNF}$ treatment (Fig. 4). The rapid shift into inedible bacterial biomass could also explain the leveling off or decrease in HNF numbers (Figs. 1, 3) by the end of the experiments.

The interpretation of the effect of the $<20 \mu\text{m}$ experimental treatments is more complicated, although the major features (i.e., shifts in bacterial cell volume, proportion of filaments, and total protistan bacterivory) resembled those found in the $<5 \mu\text{m}$ treatments. In experiment I, the shift in BCC in the $<20 \mu\text{m}$ treatment was highly significant, whereas in experiment II it was not. However, this could also reflect an important difference in the initial conditions in the two experiments—namely, grazing pressure exerted by protists on the bacterioplankton (Table 2). For example, in experiment I, the removal rate in the reservoir was only 8%, but this rate increased by almost one order of magnitude in the $<20 \mu\text{m}$ bag.

The same theory (i.e., the discrepancy between the incubation-induced, high daily removal rate of bacterial standing stock in experiment I compared with that in the ambient reservoir water) could also explain the smaller but still significant changes in BCC in the UNF treatment. The transfer of micro- and macrozooplankton from the original water sample into the dialysis bag was probably not quantitative, as was indicated by the zooplankton analysis at the end of experiment I (Table 1). Hence, there was probably little top-down control of protists dominated by bacterivorous HNF and small oligotrichs, which are also efficient picoplankton feeders (Šimek et al. 1995; Stabell 1996).

The preexisting conditions in experiment I (i.e., the minor role of protists in reservoir bacterivory) could explain our findings that in the $<1 \mu\text{m}$ treatment, BCC did not change. Thus, the transfer of bacterioplankton from ambient water into the predator-free treatment did not substantially change with respect to the low protistan grazing pressure. However, these data would also imply a negligible direct grazing effect related to nonselectively filtering metazooplankton on BCC—contrary to the finding that they often control bacterioplankton abundance (and possibly also size structure) during clear-water phases (e.g., Jürgens 1994). In the $<1 \mu\text{m}$ treatment, we did not observe changes in bacterial mean cell volume or BCC, but bacterial numbers increased when bacteria were relieved from the entire grazer plankton community. Thus, metazooplankton grazing probably controlled bacterial abundance in the reservoir water (Pace et al. 1990; Šimek et al. 1990; Jürgens 1994), but it had little effect on BCC.

In terms of plankton community composition and protistan bacterivory, experiment II offered a quite distinct scenario. Prior to the experiment, reservoir bacterioplankton incubated in the $<0.8 \mu\text{m}$ treatments were exposed to relatively high protistan grazing pressure, equivalent to the

daily removal of 31 and 55% of bacterial standing stock and production, respectively (Table 2, Fig. 3). Thus, the highly significant shift in BCC found in the $<0.8 \mu\text{m}$ treatments occurred in highly grazed populations transferred into predator-free conditions. Also, the shift in BCC was quite specific—there was a conspicuous increase in the proportion of BET and a slight increase in that of ALF in contrast with other treatments (the $<5 \mu\text{m}$ and $<5 \mu\text{m} + \text{HNF}$ treatments). In the latter treatments, the significant change in BCC was that the proportion of ALF roughly doubled, whereas BET did not show any trend, and the C/F subgroup increased slightly. Thus, the data from the $<0.8 \mu\text{m}$ treatments could exemplify a situation in which the bacterial community, relieved from selective grazing pressure exerted by protists, shows increases in average cell size (Šimek and Chrzanowski 1992), and some strains that had formerly been suppressed tend to increase their proportions, accompanied by a temporal increase in bacterial production (Fig. 3). Based on the total bacterial cell number increase between the beginning and the third day of the incubation in the $<0.8 \mu\text{m}$ treatment (Fig. 3), we calculated an overall doubling time of 60 h for this bacterioplankton community. However, changes in the proportions of the different bacterial subgroups during this period (Fig. 4) suggest that subgroups were growing at different rates. Our calculations suggest a much shorter doubling time for BET (33 h) and for ALF (47 h), in contrast to the longer doubling times of C/F and GAM (77 and 240 h, respectively). Similar grazing-induced changes in growth rates of different bacterial groups have been observed in a chemostat study (Perthaler et al. 1997b; Šimek et al. 1997).

The bacterial communities in two other treatments ($<20 \mu\text{m}$ and UNF) did not show any significant BCC shift in experiment II. These communities were exposed initially and by the end of the study to protistan grazing pressure that was similar to those rates found in the reservoir (Fig. 3, Table 2). Only in the middle of the experiment was protistan bacterivory temporarily enhanced; however, this was largely because of ciliate grazing, with a dominance of coarse filter-feeding or detritofagous species, such as *Cinetochilum margaritaceum* or *Coleps* sp. (Foissner and Berger 1996), that are not specialized, selective bacterial consumers (Šimek et al. 1995). Ciliates were likely also responsible for efficient control of HNF dynamics (Weisse et al. 1990) in the $<20 \mu\text{m}$ treatments in both experiments.

In the UNF treatment in experiment II, all the measured bacterial and protozoan parameters changed very little, which could be attributed to the stabilizing role of the present zooplankton community (with a high proportion of fine filter-feeding cladocerans, *Ceriodaphnia quadrangula* and *Diaphanosoma brachyurum* [Geller and Müller 1981], the concentrations of which did not change remarkably during incubation in the dialysis bag).

A strong increase of cell length distribution toward filamentous cells (for protists, inedible cells) of phylogenetically distinct subgroups of *Eubacteria* (Figs. 2, 4) was observed in all “grazed” treatments. The filament formation also had season-specific aspects: in experiment I, all filaments belonged to ALF; in experiment II, most of filaments hybridized with the C/F probe. In a chemostat study, this survival strategy (i.e., filament formation in the presence of a flagel-

late bacterivore) was found in the BET subgroup (Pernthaler et al. 1997b; Šimek et al. 1997). This may indicate that this mechanism represents a phylogenetically broadly distributed defense strategy against being grazed upon. On the basis of laboratory experiments, Hahn and Höfle (1998) suggested that the occurrence of filamentous cells may not be a direct response to chemical stimuli released by a protistan predator but rather a response to an increased growth rate due to grazing. Our data show a relationship in which an increased portion of filamentous bacteria corresponds to increasing protistan bacterivory.

We did not quantify the impact of viral lysis in our experiments (potentially an important source of host-specific bacterial mortality; see Suttle 1994); such an impact could have partially influenced BCC. However, since all viruses are smaller than 0.8 μm , the size fractionation of water samples cannot selectively influence either abundance or species composition of viruses in comparison with the well-defined treatments conducted with the bacterivore communities. Moreover, our data showed that no significant changes in BCC occurred when the initial removal rate of bacterial standing stock roughly persisted during the incubation in the experimental treatment. The most significant changes in BCC were primarily associated with treatments involving strong changes in protistan grazing pressure, relative to pre-incubation conditions.

However, we are aware of the limitations of our data: only rather crude morphologic and BCC shifts are detected, based on splitting the community into a few subgroups consisting of an unknown number of bacterial strains (Amann et al. 1995). Despite such limitations, we may conclude that selective protistan grazing is one of the keystone factors shaping BCC of natural bacterial assemblages. On the other hand, our knowledge, drawn from previous laboratory predator-prey chemostat models (Pernthaler et al. 1997b; Šimek et al. 1997) and from this study, already indicate certain generalities concerning major features of the relationship between bacterial prey and protistan predator. For instance, if the normal balance between protozoan grazing rate and the rate of “genome-specific removal” is violated by a sudden enhancement of grazing, only those strains able to balance their grazing losses with higher growth rates (Pernthaler et al. 1997b) or, alternatively, those able to develop some grazing-resistant strategy (Jürgens and Güde 1994; Šimek et al. 1997) will maintain their presence in the community. Moreover, some of them can even profit from the new situation by occupying niches vacated by strains whose physiological capabilities were not sufficient in the new, strongly top-down-controlled conditions. In contrast, a sudden relief from grazing pressure (see Fig. 4, <0.8 μm treatment) will induce a strong shift in BCC that is caused (1) by an increasing proportion of the formerly selectively grazed strains or (2) by the decreasing proportion of highly active strains that previously profited from the positive feedback of bacterivory (i.e., low numbers of bacteria along with a high substrate and nutrient availability mediated by grazing) (Sherr et al. 1982; Blöem et al. 1989).

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