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Morphological and Compositional Shifts in an Experimental Bacterial Community Influenced by Protists with Contrasting Feeding Modes

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In a two-stage continuous-flow system, we studied the impacts of different protozoan feeding modes on the morphology and taxonomic structure of mixed bacterial consortia, which were utilizing organic carbon released by a pure culture of a *Rhodomonas* sp. grown on inorganic medium in the first stage of the system. Two of three second stages operated in parallel were inoculated by a bacterivorous flagellate, *Bodo saltans*, and an algivorous ciliate, *Urotricha furcata*, respectively. The third vessel served as a control. In two experiments, where algal and bacterial populations grew at rates and densities typical for eutrophic waters, we compared community changes of bacteria, algae, and protozoa under quasi-steady-state conditions and during the transient stage after the protozoan inoculation. In situ hybridization with fluorescent oligonucleotide probes and cultivation-based approaches were used to tentatively analyze the bacterial community composition. Initially the cell size distribution and community structure of all cultivation vessels showed similar patterns, with a dominance of 1- to 2.5-μm-long rods from the beta subdivision of the phylum *Proteobacteria* (β-Proteobacteria). Inoculation with the ciliate increased bacterial growth in this substrate-controlled variant, seemingly via a recycling of nutrients and substrate released by grazing on algae, but without any detectable effect on the composition of bacterial assemblage. In contrast, an inoculation with the bacterivore, *B. saltans*, resulted in a decreased proportion of the β-Proteobacteria. One part of the assemblage (<4% of total bacterial numbers), moreover, produced large grazing-resistant threadlike cells. As *B. saltans* ingested only cells of <3 μm, this strategy yielded a refuge for ~70% of total bacterial biomass from being grazed. Another consequence of the heavy predation in this variant was a shift to the numerical dominance of the α-Proteobacteria. The enhanced physiological status of the heavily grazed-upon segment of bacterial community resulted in a much higher proportion of CFU (mean, 88% of total bacterial counts) than with other variants, where CFU accounted for ~30%. However, significant cultivation-dependent shifts of the bacterial community were observed toward γ-Proteobacteria and members of the Cytophaga/Flavobacterium group, which demonstrated the rather poor agreement between cultivation-based approaches and oligonucleotide probing.

Substrate supply and grazing have been considered principal factors controlling bacterial growth in natural aquatic systems (53). Protozoa, especially heterotrophic nanoflagellates and ciliates (with the ability to graze on a variety of bacterial species), are usually the major consumers of bacteria in water environments (for examples see references 25 and 32). Apart from regulating abundance, however, protozoa may also influence the bacterial community structure. Size, shape, and cell wall and surface-specific properties of bacteria play important roles in the selection of bacterial prey and thus result in different mortalities of bacterial morpho- and genotypes.

A protozoan grazing on bacteria is size selective (7, 12, 20, 39, 42), and protozoa can partly control bacterial standing stock abundance by preferentially removing dividing cells (12). Selective grazing of more rapidly growing larger cells may also explain, in part, the ability of small cells to escape predation and persist in bacterioplankton assemblages (7, 35, 39). Besides, protozoa consume and digest various bacterial species with differential efficiencies (11, 19).

The most frequently observed consequence of heavy protozoan grazing pressure in natural pelagic environments is a dominance of rather small bacterioplankton cells accompanied by the high total secondary productivity of the community (for examples see references 27 and 38). Only those strains capable of balancing grazing losses and growth rate are likely to be present in such assemblages, and thus protozoan grazing also influences physiological properties of the bacterial populations. From the systems, where protozoa were responsible for most of bacterial mortality, there is evidence that protozoan grazing and bacterial production are positively correlated (8, 30, 31). Bacterial abundance under grazer control is usually moderate to low, and bacterial specific growth rate is high (see model by Wright [53]), compared with the opposite situation of substrate control (low grazing pressure), which results in high bacterial density but low specific growth rates. This implies that under grazing control a high cell-specific growth rate (53) and/or high specific metabolic activity of bacteria can be expected, a phenomenon supported by the finding of a tight positive correlation of total protozoan bacterivory versus the proportion of metabolically active or the cell-specific activity of respiring bacteria (24, 41).

Alternatively, under conditions of heavy protozoan grazing, the distribution of bacterial cell types may shift toward cells resistant to grazing by forming spirals (13) or aggregates (37) or cells with prosthecae (3). Thus selective protozoan bacterivory should favor particular, rapidly reproducing, or grazing-resistant bacterial species or populations (11, 18, 39), resulting in shifts in bacterial community composition. Recent findings

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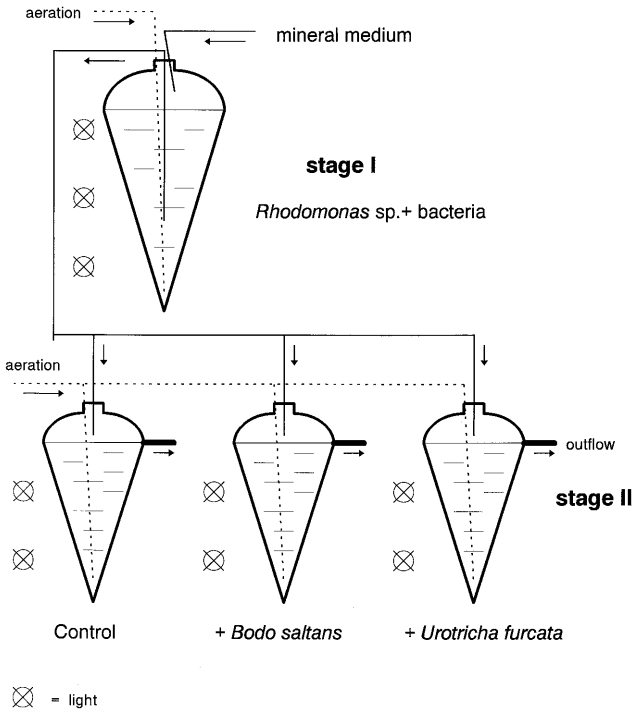

FIG. 1. Diagram of the system arrangement used in both experiments.

by Höfle (17) at least partly supported this hypothesis. Using direct analysis of RNA in bacterioplankton, he showed that high genotypic diversity during the spring algal bloom was reduced significantly during intensive bacterial grazing by heterotrophic nanoflagellates.

The principal question is to what extent the frequently observed morphological and physiological changes only exemplify the phenotypic plasticity of bacterial strains and to what extend they are linked to genotypic changes in the community composition. Natural aquatic ecosystems are of high complexity, so that it is difficult to test these hypotheses directly in situ with reproducible results. Therefore, we have designed a two-stage continuous-flow system in which an algal monoculture with the accompanying microbial consortia was produced in the first stage with abundances and growth rates typical for eutrophic waters. The second stages of the system were inoculated by protozoa of contrasting feeding modes. The aim of the study was to determine the impacts of a typical bacterivorous flagellate and algivorous ciliate on bacterial abundance, morphology, and community structure in such a simplified experimental system.

MATERIALS AND METHODS

Experimental design of continuous-flow systems. We ran two almost identically designed continuous-flow systems assigned as experiments 1 and 2, both consisting of four experimental vessels arranged in two cultivation stages (for details, see Fig. 1). At the first stage of the system (referred to as stage I) we used a 2-liter conical vessel, into which slightly modified WC (MBL [14]) medium was pumped. Algal growth was limited by phosphorus (final concentration, 70 μg liter^{-1} PO_4–P). All components of the medium as well as cultivation vessels were sterilized. Stage I (volume, 1.5 liter) was aseptically inoculated with a monospecific stock culture of an alga, a *Rhodomonas* sp., and the accompanying mixed assemblage of bacteria which developed along with the algal culture. Dilution rates in stage I of the system were adjusted to 0.42 and 0.46 day^{-1} in experiments 1 and 2, respectively.

After 3 to 4 days, when growing algae in stage I reached an abundance of ~5 × 10^4 ml^{-1}, the algal culture with the accompanying microflora was pumped into three parallel second-stage vessels (0.5-liter conical vessels filled to 0.47 liter) with dilution rates of around 0.19 day^{-1} in experiment 1 and 0.25 day^{-1} in experiment 2. One of the vessels served as the control (assigned as II/control). When steady-state algal growth was reached in the second stages, protozoa with different feeding modes were inoculated into the remaining two experimental vessels. The second vessel (assigned as II/*Bodo*) was inoculated with a bacterivorous flagellate, *Bodo saltans*, a protist grazing on the accompanying bacteria but unable to ingest algal cells. The third vessel (assigned as II/*Urotricha*) was inoculated with an algivorous ciliate, *Urotricha furcata* (Fig. 1).

B. saltans was maintained on wheat seed infusion (one seed autoclaved in 100 ml of tap water) with a mixed assemblage of bacteria and *U. furcata* was maintained on a pure *Rhodomonas* sp. culture growing with accompanying bacteria in the same medium used for both experiments. Prior to the protozoan inoculation, flagellates and ciliates from maintenance cultures were gently concentrated from 25 to about 5 ml by gravity filtration in an autoclaved filter holder on 2- or 5-μm-pore-size filters, respectively. The protozoa were washed five times (gravity filtration) with 20 ml of autoclaved bacteria-free (0.2-μm-pore-size filters) tap water (flagellates), or with the WC medium (ciliates). This procedure removed most bacteria originally present in the maintenance cultures (49). Ciliates contained in the upper 2 ml of the remaining 6 to 8 ml of the last wash in the filter funnel were aseptically inoculated into the II/*Urotricha* variant.

A specific treatment was used to minimize the possibility of the bacterial contamination of the II/*Bodo* vessel when it was inoculated with *B. saltans*. The flagellates contained in the upper 2 ml of the last wash in the filtration funnel (no bacteria observed via epifluorescent microscopy) were aseptically inoculated into 50 ml of a 2-μm filtrate of the subsample from stage I containing bacteria only. Flagellates were allowed to adapt to and grow on these bacteria for 30 h, and then two additional runs of the above wash and reinoculation procedure were conducted. After the last wash with this treatment, flagellates contained in 2 ml of the bacterium-free subsample from the remaining ~8 ml were aseptically inoculated into the II/*Bodo* experimental vessel.

All experimental vessels were operated at 20°C as a cyclostat: under a light-dark regime of 16 h of light (160 microeinsteins m^{-2} s^{-1}) and 8 h of darkness (for details see reference 50) and continuously aerated and mixed by fine bubbling from the bottom of vessels. The only exception was the II/*Urotricha* variant, for which we used 1-min bubbling pulses every 20 min to avoid a possible interference of the strong water currents created by aeration in the conical vessels with the feeding mode of this raptorial ciliate. Preliminary tests with *U. furcata* indicated its inability to grow in a continuously bubbled vessel.

In pilot experiment 1, we measured abundance of algae, bacteria, and protozoa and grazing upon the bacteria during the quasi-steady state for 30 days (Fig. 2). In experiment 2, we studied impacts of the protozoan grazing through the transient stage immediately following the flagellate and ciliate inoculation (second day of the 12-day study period) into the II/*Bodo* and II/*Urotricha* variants, respectively. The same parameters as in experiment 1 were monitored (Fig. 3), but a detailed analysis of bacterial size structure and morphology and a tentative identification of dominant bacterial groups in all variants were performed.

Bacterial abundance and biomass. Subsamples were fixed with formaldehyde (2% final concentration), stained with DAPI (4′,6-diamidino-2-phenylindole) (final concentration, 0.2% [wt/vol]), and enumerated by epifluorescence microscopy (Olympus BH2). Between 400 and 600 bacteria were sized by semiautomatic image analysis (Lucia, Laboratory Imaging, Prague, Czech Republic). For more details see Psenner (26) and Šimek et al. (38). Bacterial biomass was calculated according to the allometric relationship between cell volume and carbon content (22). As cell length was the most important factor determining the ability of *B. saltans* to ingest bacterial cells and bacterial width showed little variability, we chose the variation in cell length to subdivide bacteria into seven size classes at 0.4-μm intervals (for details, see Pernthaler et al. [24]).

Protozoan and algal abundance and flagellate grazing on bacteria. Subsamples were fixed by adding 0.5% of alkaline Lugol's solution immediately followed by formaldehyde (2% final concentration) and several drops of 3% sodium thiosulfate to clear the Lugol's color (33). Two- to five-milliliter (*B. saltans* and *Rhodomonas* sp.) or 5- to 20-ml (*U. furcata*) subsamples stained with DAPI were filtered through 1-μm black Poretics filters and enumerated by epifluorescence microscopy. To estimate grazing rates of *B. saltans* on bacteria, we produced fluorescently labelled bacteria (FLB) from the accompanying bacterial microflora growing together with the algae in the first stage of the system. Bacteria were concentrated and fluorescently labelled according to the protocol of Sherr and Sherr (33).

We conducted short-time FLB direct uptake experiments in 70-ml subsamples from the II/*Bodo* vessel where the added tracer amount constituted 8 to 15% of bacterial abundance. To determine flagellate ingestion rates, 15-ml subsamples were taken at time series of 10, 20, and 30 min after tracer addition and preserved according to Sherr and Sherr (33). Samples from zero time were also inspected to avoid potential bias due to attachment of noningested FLB on flagellate surfaces. To estimate total protozoan grazing, we multiplied average uptake rates of flagellates by their abundances.

FISH. In experiment 2, a tentative analysis of bacterial community structure was carried out by in situ hybridization with fluorescent oligonucleotide probes (FISH) on membrane filters (1). The five different oligonucleotide probes were targeted to the kingdom Bacteria, (EUB), the alpha, beta, and gamma subdivisions of the phylum *Proteobacteria* (α-, β-, and γ-*Proteobacteria*) (ALF, BET, and GAM), and at the *Cytophaga*/*Flavobacterium* group (CF) (2). The probes were

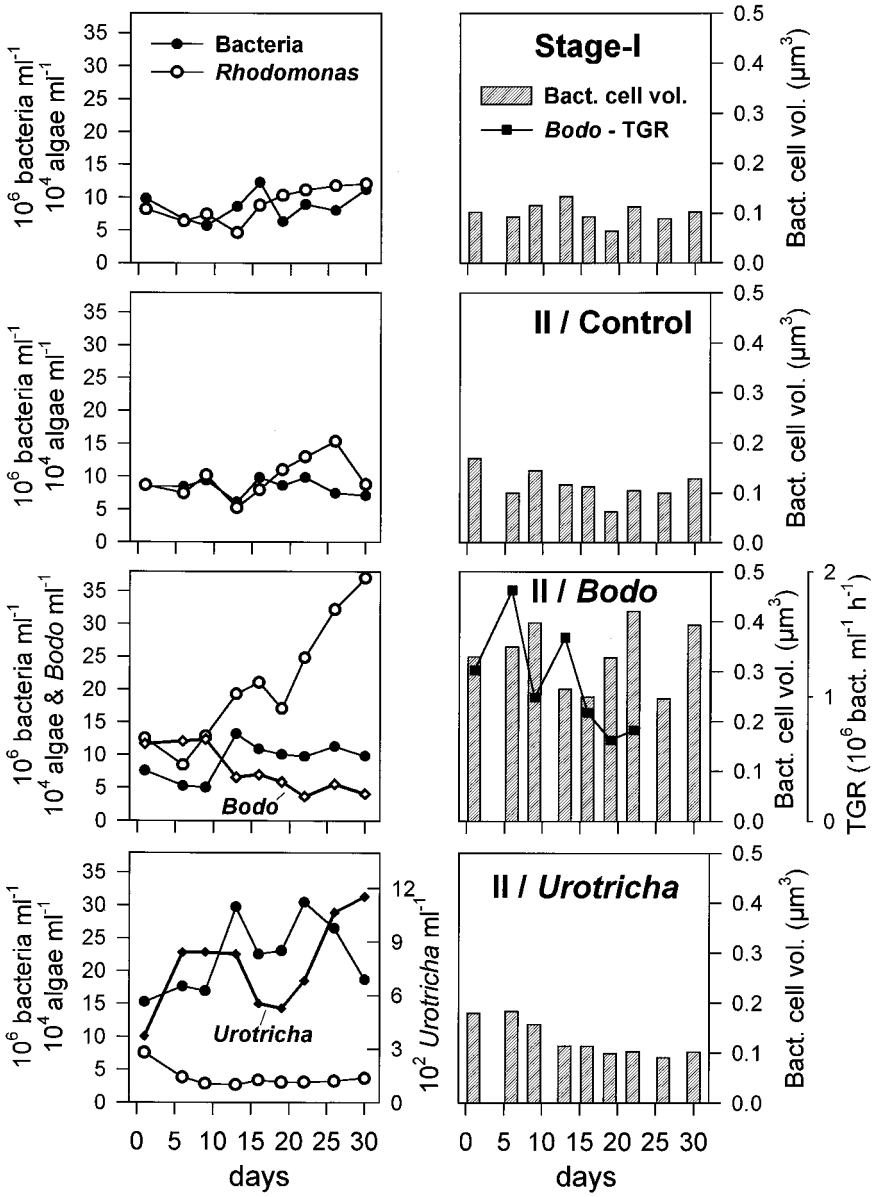

FIG. 2. Results of experiment 1. (Left panels) Abundances of bacteria, algae, and protists in the different variants of the continuous-flow system. (Right panels) Bacterial mean cell volumes in all variants and total grazing rates (TGR) of *B. saltans* on bacteria in the II/*Bodo* variant.

fluorescently labelled with the indocarbocyanine dye CY3 (BDS, Pittsburgh, Pa.). Subsamples from all dates and variants were fixed on membrane filters according to Alfreider et al. (1) and stored at −20°C. Sections cut from these filters were hybridized with EUB, ALF, BET, GAM, and CF and stained with DAPI, and the percentage of hybridized bacterial cells was enumerated by epifluorescence microscopy. For details of the hybridization procedure and error estimates see Pernthaler et al. (23).

Cultivation and identification of bacterial strains. During experiment 2, bacteria were cultivated on Nutrient Broth No. 2 Agar (NBA; Imuna Šarišské Michal'any, Slovakia) plates from all variants at each sampling day. CFU were read on NBA plates after a 7-day incubation at 23°C. According to morphological characteristics of colonies, five to six typical groups were usually distinguished in each variant and the number of the typical colonies within each group was determined. The typical representatives of the colonies were reinoculated on NBA plates and all growing strains were later identified with standard biochemical identification kits (NLF-test; Lachema, Brno, Czech Republic). The results were evaluated using the TNW microbiological identification system by Pavel Benda distributed via the Czech Collection of Microorganisms (CCM, Brno, Czech Republic).

RESULTS

Continuous-flow systems. Basically, the same parameters, measured in both experiments, showed somewhat similar patterns (cf. Fig. 2 and 3). In experiment 1, a quasi-steady state of algal and bacterial growth monitored for 30 days was characterized by slightly fluctuating abundances of bacteria (~5×10^6 to 13×10^6 cells ml^{-1}) in all but the II/*Urotricha* variant where numbers of bacteria ranged from 15×10^6 to 30×10^6 cells ml^{-1} (Fig. 2). The abundance of the *Rhodomonas* sp. in both stage I and II/control ranged from ~5×10^4 to 15×10^4 cells ml^{-1}. In the II/*Bodo* variant the algal numbers increased conspicuously, from $<10 \times 10^4$ to $>36 \times 10^4$ cells ml^{-1} throughout the study period, while in the II/*Urotricha* variant they were mostly well below 5×10^4 cells ml^{-1}. The numbers of *B. saltans* showed an opposite trend: they decreased from

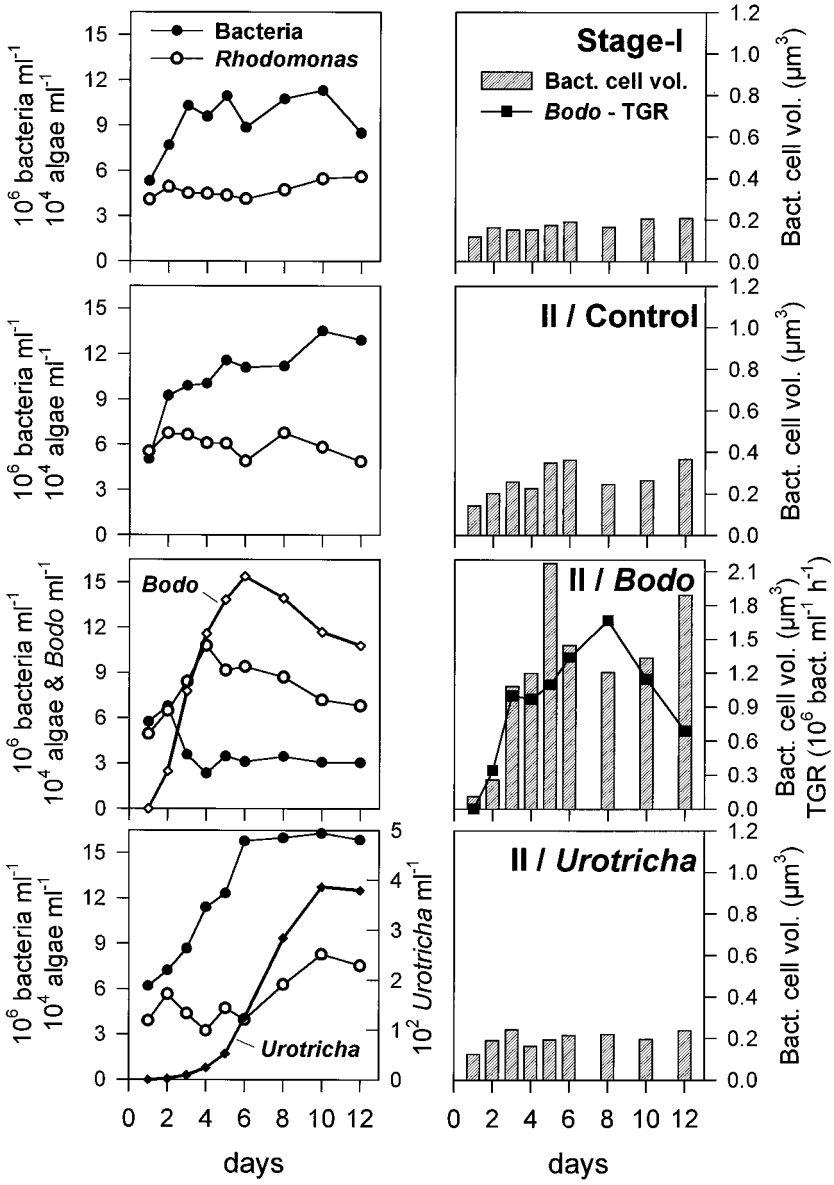

FIG. 3. Results of experiment 2. (Left panels) Abundances of bacteria, algae, and protists in the different variants of the continuous-flow system. (Right panels) Bacterial mean cell volumes in all variants and total grazing rates (TGR) of *B. saltans* on bacteria in the II/*Bodo* variant.

$\sim 12 \times 10^4$ cells ml^{-1} during the first 10 days to about 4×10^4 to 7×10^4 cells ml^{-1} for the rest of the study (Fig. 2). The whole flagellate population exerted a very strong total grazing pressure (0.7×10^6 to 1.85×10^6 bacteria ml^{-1} h^{-1}) on the bacterial assemblage (cf. also experiment 2). Flagellate grazing strongly shifted the size class distribution of bacterial cells (data not shown) toward an increased proportion of filamentous bacteria. It resulted in significantly larger mean cell volumes of bacteria in the II/*Bodo* variant than in other variants (paired *t* test, $P \leq 0.028$, $t_d = 2.66$ to 5.05, $df = 8$). In contrast, the differences in the mean cell volume among the stage I, II/control, and II/*Urotricha* variants were insignificant (paired *t* test, $P > 0.05$, $t_d = 1.27$ to 1.37, $df = 8$) and their bacterial assemblages were dominated by relatively uniform, mostly rod-shaped cells with a mean volume of 0.07 to 0.185 µm^3.

In experiment 2 (Fig. 3), we monitored the transient stage following protistan inoculation into the system. Bacterial abundances in stage I and II/control showed a slight increase during the first 5 days and then stabilized between $\sim 8 \times 10^6$ and 13×10^6 cells ml^{-1}. Algal abundances were fairly stable throughout the period, ranging between $\sim 4 \times 10^4$ and 7×10^4 cells ml^{-1}. Both bacterial and algal abundances kept the same ranges of values during 12 days (not shown) following the period depicted in Fig. 3.

In the II/*Bodo* variant, in parallel with increasing flagellate numbers, bacteria dropped from $\sim 7 \times 10^6$ to 3×10^6 cells ml^{-1} in contrast with increasing *Rhodomonas* sp. numbers during the first 4 days, and then both remained relatively stable. However, in general the abundances of bacteria and algae showed an inverse trend ($r^2 = 0.513$, $n = 9$, $P < 0.02$). *B. saltans* abundance increased sharply to 16×10^4 cells ml^{-1} during the first 6 days, then it slowly decreased to $\sim 10 \times 10^4$

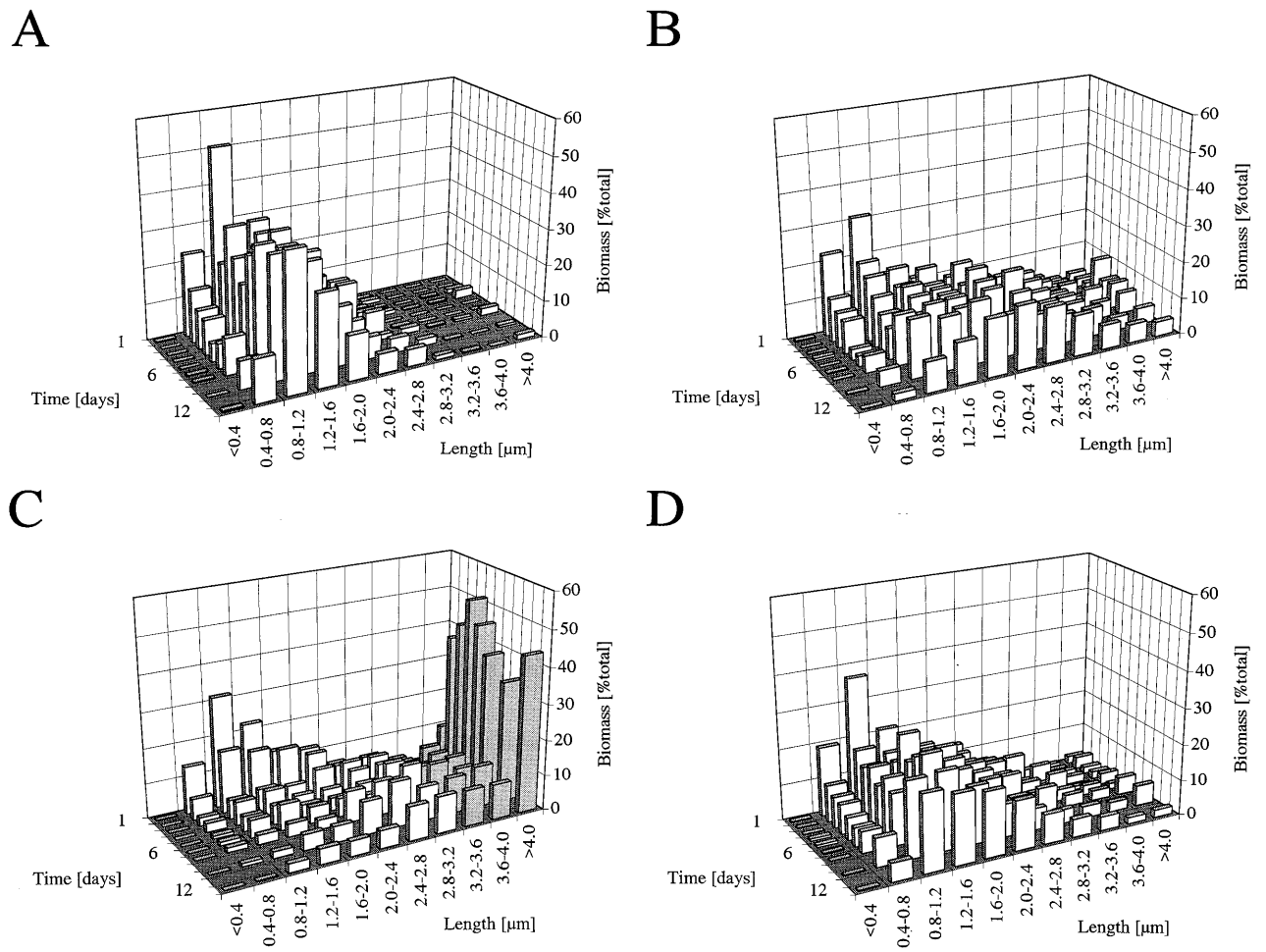

FIG. 4. Distributions of bacterial biomass (percentages of total bacterial organic carbon) in different size classes (cell length) in the stage I (A), II/control (B), II/*Bodo* (C), and II/*Urotricha* (D) variants. Shaded bars, protist-inedible parts of the bacterial biomass.

cells ml^{-1} (Fig. 3), and this abundance also remained roughly stable for the 12 days following the period depicted in Fig. 3 (data not shown).

In the II/*Urotricha* variant, ciliate numbers sharply increased up to ~400 cell ml^{-1} without exerting any clear effect on algal abundance, which ranged from 3.3×10^4 to 7.5×10^4 cells ml^{-1} (Fig. 3). On the other hand, probably due to nutrient and carbon recycling as a consequence of ciliate grazing upon algae, bacterial numbers increased in parallel with the number of ciliates, yielding a highly significant correlation between their abundances ($r^2 = 0.715$, $n = 9$, $P < 0.01$).

On the first day, i.e., prior to the protistan inoculation into the two experimental variants, the mean cell volumes of bacteria were very similar in all variants (between 0.1 and 0.15 μm^3), and mostly cells of the size classes between 0.4 to 1.6 μm for cell length clearly dominated the total bacterial biomass (Fig. 3 and 4). During the following days we observed different patterns. Comparing to those in stage I, bacterial mean cell volumes in the II/*Urotricha* variant (Fig. 3) were slightly but significantly different (paired t test, $P < 0.05$, $t_d = 2.78$, $df = 8$) and total biomass was mainly made up of bacteria of between 0.4 and 1.6 μm in cell length. Basically, the inoculation of the vessel with algivorous *U. furcata* had very little effect on the size structure of the bacterial assemblage. In comparison with stage I we observed only slightly increased contributions of cells within size classes of >2 μm, which together accounted for only 20 to 30% of total bacterial biomass (cf. Fig. 4A and D). The pattern of the size class distribution in the II/control variant was very similar to that observed in the stage I and II/*Urotricha* variants (cf. Fig. 4B and A and D), but the mean cell volume was significantly larger (Fig. 3; paired t test, $P < 0.02$, $t_d = 4.89$ and 3.52, respectively, $df = 8$). Also, the contributions of cells longer than 2 μm accounted for 25 to 45% of total biomass, with higher values observed in the second part of the experiment (Fig. 4B).

A quite different pattern of the size class distribution of bacterial cells was found in the II/*Bodo* variant, characterized by a remarkable shift from small toward long filamentous bacteria in the presence of the bacterivore (ingesting 8 to 14 bacteria $cell^{-1}$ h^{-1}). Within a 2-day transient stage following flagellate inoculation into the variant, in parallel to the increasing total grazing pressure of *B. saltans* upon bacteria (Fig. 3), the biomass of bacterial assemblage became dominated by large filamentous bacteria (cell length, >3.2 μm [Fig. 4C]). This shift was directly linked to the increased grazing effect of *B. saltans*, as we found a linear relationship between total grazing rate of the flagellate and the mean cell volume of bacteria throughout the experiment ($r^2 = 0.445$, $n = 9$, $P < 0.05$). When we used only a reduced data set from the first 6 days when abundance of *B. saltans* increased in parallel with the total grazing pressure exerted by the flagellate, this relationship was much tighter ($r^2 = 0.749$, $n = 6$, $P < 0.02$). Size

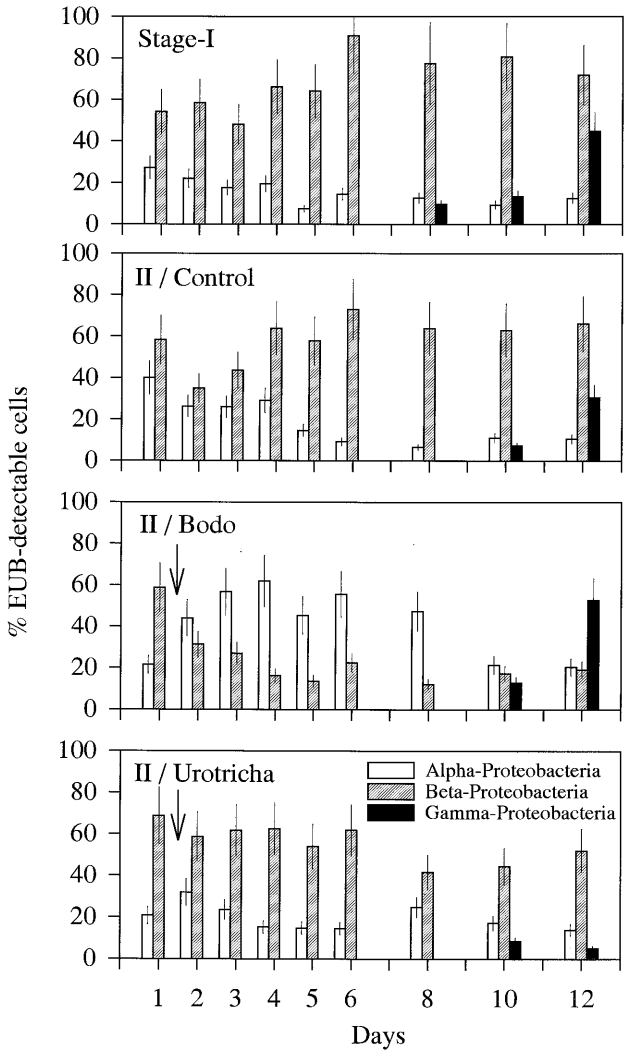

FIG. 5. Proportions of α-, β-, and γ-Proteobacteria of total EUB-detectable bacterial cells in all variants of the continuous-flow system. Arrows indicate the protozoan inoculation.

measurements of ~100 ingested FLB revealed that *B. saltans* was only able to feed on cells shorter than ~2.8 to 3 μm.

Oligonucleotide probes, cultivation, and tentative identification of bacterial strains. In experiment 2, we searched for a taxonomic background for the observed shifts in the morphology and size class distribution of bacteria (Fig. 3 and 4). FISH indicated that the remarkable morphological shifts in the II/*Bodo* variant were accompanied by corresponding changes in the community composition of bacteria (Fig. 5). In our continuous-flow system, the detectability of bacteria with the probe for *Eubacteria* (EUB) was usually between 80 and 97% of all DAPI-stainable particles (23). At the beginning, bacterial assemblages in all variants were numerically dominated by β-Proteobacteria accounting for about 60% of EUB-detectable bacterial cells. However, immediately after the inoculation with *B. saltans* into the vessel, α-Proteobacteria dominated this bacterial community for the next 7 days (~50 to 65% of EUB-detectable cells; Fig. 5). Afterwards, they decreased to about 20% of EUB-detectable cells. In contrast, the inoculation with the algivorous ciliate into the II/*Urotricha* variant did not result in any remarkable shift in the community composition of bacteria throughout the experiment, regardless of the increasing total bacterial numbers by a factor of about 2.5 (Fig. 3). By the end of the experiment some additional changes in the system were indicated by the appearance of an increasing proportion of γ-Proteobacteria in all variants, presumably starting from stage I (Fig. 5). The fraction of bacteria belonging to the *Cytophaga/Flavobacterium* group was very low throughout the experiment (<0.5%).

CFU on NBA plates usually accounted for about 30% (Table 1), ranging from 17 to 57% of the total DAPI-stainable bacterial counts in all but one variant. However, a significantly higher share of CFU (mean, 88%; range, 35 to 137%) was found in the variant with the bacterivore (II/*Bodo*). In stage I, five characteristic morphological colony types were usually distinguished, accounting together for 56% of the total CFU. The same five groups plus another group were found in both II/control and II/*Urotricha*, accounting together for 63 and 69% of the total CFU, respectively (Table 1). In II/*Bodo*, however, the fifth group was apparently replaced by another colony type (group 6 in Table 1).

We isolated a total of 132 colonies (typical representatives of each group) from all variants within 9 days, and cultivable isolates were identified. All strains tested within the first and second groups were determined as *Flavobacterium mizutaii* (4 to 5 mm, yellow colonies, $n = 12$) and *F. indologenes* (<1 mm, yellow colonies, $n = 8$), respectively. They were found in all variants, although in very reduced numbers in II/*Bodo* ($\leq 10^4$ CFU ml^{-1}; Table 1). Very tiny (<1 mm) white colonies were pooled into the third group. From these colonies ($n = 30$), the following strains were more frequently determined: *Acinetobacter lwoffii* (9), *Psychrobacter immobilis* (6), and *Kingella kingae* (3). The fourth group was formed by very mucous, 2- to

TABLE 1. Results of experiment 2[a]

Variant (*n*)	DAPI (10^6 cells ml^{-1})	CFU (10^6 cells ml^{-1})	CFU (% of DAPI)	% CFU								
				Gr. 1[b]	Gr. 2[c]	Gr. 3	Gr. 4[d]	Gr. 5[d]	Gr. 6[d]	Gr. 7	Gr. 8	Sum
Stage I (8)	9.25 ± 1.80	2.57 ± 0.87	27 ± 6	11 ± 6	11 ± 6	8 ± 3	5 ± 6	21 ± 16	NF[e]	NF	NF	56 ± 17
II/Control (8)	10.50 ± 2.33	3.04 ± 0.72	31 ± 12	8 ± 5	8 ± 5	29 ± 13	3 ± 3	11 ± 11	NF	4 ± 4	NF	63 ± 17
II/*Bodo* (7)	3.61 ± 1.25	2.91 ± 0.58	88 ± 27	0 ± 1	1 ± 6	38 ± 11	7 ± 19	NF	35 ± 21	NF	NF	82 ± 26
II/*Urotricha* (7)	12.95 ± 3.37	3.26 ± 0.67	26 ± 6	9 ± 6	4 ± 2	28 ± 21	2 ± 2	11 ± 11	NF	NF	6 ± 5	69 ± 11

[a] Total bacterial number (DAPI), CFU on NBA, the proportion of eight phenotypic groups distinguished according to characteristics of colonies on NBA, and the total proportion of groups (Gr.) 1 through 6 (sum percentages of CFU) in all variants (means ± standard deviations; the first day, i.e., before protozoan inoculation, excluded from the evaluation in both variants with protozoa) are shown.
[b] Determined as *F. mizutaii* in all cases.
[c] Determined as *F. indologenes* in all cases.
[d] Determined as *A. lwoffii* in all cases.
[e] NF, not found on plates.

3-μm cream-white colonies, which showed extremely poor growth after reinoculation. Thus only 2 of 14 strains were identified successfully as *A. lwoffii* (both from II/*Bodo*). Small (2 to 3 mm) cream-white colonies with a yellowish center were pooled into the fifth group. These strains also showed poor culturability after reinoculation, and only three strains were determined, all as *A. lwoffii*. In the II/*Bodo* variant, another colony type (2 to 3 mm, cream-white, growing well after reinoculation; group 6 in Table 1) was always determined as *A. lwoffii* ($n = 5$). Large (5 to 6 mm), cream-white colonies were isolated from II/control (group 7, Table 1) and were identified as *A. lwoffii* (2 strains), *P. immobilis* (2 strains), or *K. kingae* (2 strains). Another group (1 to 2 mm, clear, yellowish colonies) in the II/*Urotricha* variant (group 8, Table 1) showed poor growth, so only one out of seven strains was determined, again as *A. lwoffii*.

Altogether only 54% of the isolated strains were successfully identified. Twenty-two isolates were determined as *A. lwoffii*. However, many other similar strains from those groups (especially groups 4, 5, and 8) failed to grow sufficiently to allow a final determination. On the other hand, well-growing isolates of *A. lwoffii* were found in the II/*Bodo* variant (group 6). Some strains forming typical filaments were also isolated from mucous colonies (group 4, Table 1), but they could not be determined via the standard cultivation techniques. Because of their conspicuous morphology, we could microscopically confirm that these strains were the same that dominated bacterial biomass in the II/*Bodo* variant. Frequently identified strains from groups 3 and 7 were *P. immobilis* (8 strains) and *K. kingae* (5 strains).

DISCUSSION

We designed the continuous-flow system to mimic roughly abundances and growth rates of bacteria and algae typical for the period of the spring phytoplankton peak in meso- and eutrophic waters (for examples see references 44 and 52). However, we must face the criticism of this approach by Brock (5), that our study was carried out "at some ill-defined stage" of microbial succession in the continuous culture system and that we thus used a "mixed culture of unknown provenance." Indeed, some authors described predator-prey microcosms containing several well-defined coexisting bacterial strains (15, 48) but gave no information as to whether the investigated bacterial species also form communities under natural conditions. We know of numerous studies that use single bacterial strains as protistan prey (e.g. references 16, 19, 29, 46, 47, and 49). On the other hand, enrichment cultures and filtrates from lake or sea water have been used as "natural" bacterial populations by microbial ecologists (for examples, see references 4, 12, 34, and 42).

Our work attempts to bridge the gap between these approaches. As it is not quite known which are the important bacterial species in aquatic communities, we have constrained the studied microbial community by one well-defined and reproducible factor, i.e., the carbon release by a *Rhodomonas* sp. at a given growth rate and nutrient supply. Photosynthetically produced dissolved organic carbon is an important substrate for natural aquatic bacterial communities (6, 9). Furthermore, in both experiments we used inorganic medium, and no algal mortality in stage I was observed. Thus algal exudates must have been the principal carbon source for the accompanying bacterial microflora dominated by β-Proteobacteria (Fig. 5). In fact, members of the same subdivision were also found to dominate in the bacterioplankton of oligo- to mesotrophic lakes (1, 10), where algal exudates might be of high importance for bacterial growth (6). From the chemostat theory we deduced that in the II/control variant nutrients in the medium were substantially depleted. However, almost the same pattern of size class distributions and the ratio between α- and β-Proteobacteria were found in stage I and II/control and, surprisingly, also in the II/*Urotricha* variant (cf. Fig. 4A, B, and D and Fig. 5).

We did not measure directly ciliate grazing on the *Rhodomonas* sp., but almost every ciliate individual was observed with 4 to 10 food vacuoles containing algae at various degrees of digestion, and bacterial biomass concentration increased in the II/*Urotricha* variant (Fig. 3). As bacterial abundance was very tightly correlated with ciliate abundance, the enhanced bacterial production likely resulted from carbon and nutrient recycling as a consequence of ciliate algivory. The data clearly indicate that the inoculation with this algivorous ciliate, representing a typical raptorial feeder with a negligible capture efficiency on picoplankton (40), resulted only in an increased bacterial production, but it did not have any detectable impact on the community and size structures of the bacterial assemblages, which remained dominated by 40 to 60% of β-Proteobacteria. Thus, the microbial community composition, at least at the level of subdivisions of the phylum *Proteobacteria*, was not significantly affected by a moderate change in substrate supply in the absence of a bacterivorous predator.

In contrast, a quite different scenario was represented by the II/*Bodo* variant, where a clear shift from β- to α-Proteobacteria was observed (Fig. 5). This is evidence that in the presence of a bacterivorous grazer the composition of a mixed bacterial community can be shifted within 1 to 2 days. As a result of heavy protozoan grazing some strains formed threadlike cells (see Fig. 4C in this study and Fig. 4 in reference 23). Pernthaler et al. (23) showed that the majority of filamentous bacteria belonged to β-Proteobacteria. Our inspection of food vacuole contents of *B. saltans* revealed that the cell length of ~3 μm was the upper limit of ingestable food particles for the flagellates. Within 3 days after flagellate inoculation, the protist-inedible bacteria (>3 μm), morphologically absent in the parental bacterial community, constituted 60 to 75% of total bacterial biomass (Fig. 4C). This phenomenon clearly demonstrated the great ecological effectiveness of this defense strategy. We observed a steep biomass increase of protist-inedible bacteria within 2 days, which implies a rapid cell elongation during the formation process of those filaments. Afterwards, however, the biomass of protist-inedible cells was constant during the remaining study period, indicating a low growth rate (given by the dilution rate, 0.25 d^{-1}) of this bacterial fraction. The latter finding might partly explain that this morphological shift had little effect on the culturability of the filamentous bacteria. Several times we isolated the strains with the typical filamentous morphology, but due to their low viability on cultivation media we did not succeed in identifying them. This corresponds well to the finding that the same strain, the parental cells of which were typically short rods, produced elongated, slowly growing cells when exposed to ciliate grazing (21, 36). The formation of grazing-resistant bacterial populations also seems to be an opportunistic strategy of the slowly growing segment of a natural bacterioplankton community, usually observed during periods with a low abundance of filter-feeding zooplankton (18).

Another successful survival strategy within β-Proteobacteria seemed to be determined by a flagellate selectivity against some of the strains that were within the edible size range (1 to 2 μm) of *B. saltans* (23). Selectivity against these members of β-Proteobacteria might have been linked to cell wall and sur-

face-specific properties of these strains (11) or to their nutritional inadequacy and motility (19).

The measured grazing rates (~0.8×10^6 to 1.7×10^6 bacteria ml^{-1} h^{-1}) and bacterial numbers (~3×10^6 to 6×10^6 bacteria ml^{-1}) in the II/*Bodo* variant (Fig. 3) yielded the average doubling time of the bacteria to be ~2 to 4 h. Since a significant proportion of bacterial cells were protist inedible, this sets an upper limit of the doubling time of the cells vulnerable to predation, implying thus their extremely high growth rate and cell-specific activity. In fact, Pernthaler et al. (23) proved that this was the case for one or several genotypes of α-Proteobacteria in this study. Soon after the flagellate introduction into the system, α-Proteobacteria numerically dominated in this strongly grazing-affected bacterial assemblage (Fig. 5). In parallel, however, they were ingested much faster than would correspond to their proportion within the community (23), indicating a very tight predator-prey linkage. These strains of relatively uniform morphology (1- to 2-μm-long rods) represented the only rapidly reproducing segment of the bacterial population, balancing almost the entire grazing losses by increased growth rates (23), likely at the cost of available substrate and nutrients released through flagellate bacterivory. Moreover, in our system limited by available phosphorus, bacterivory could also recycle a significant part of phosphorus bound in bacterial biomass (28), stimulating thus the additional growth of the *Rhodomonas* sp. (Fig. 2 and 3).

On the basis of our experiments we can extend the model of short-term control of bacterioplankton by substrate and grazing (53) by including a hypothesis about the potential impacts of protozoan bacterivory on community composition of mixed natural bacterial assemblages. Under strong protozoan grazer control, all the characteristics of bacterial growth rate and activity are expected to be high, especially in terms of the cell-specific activity (4, 24, 41). When bacterial strains are not able to produce morphotypes resistant to grazing as filaments or flocs or those which are less digestible (11), then significant selection of the fast-growing bacterial strains resulting in shifts in the bacterial community composition could be expected.

The quick response of α-Proteobacteria to flagellate inoculation into II/*Bodo* (Fig. 5) clearly exemplifies such a bacterial community under strong grazing control, i.e., bacteria with smaller cell volumes (vulnerable to the predation) with an extremely high calculated growth rate (for details see reference 23) as well as with high physiological activity (Table 1). This status was indirectly indicated also by three to seven times higher bacterial cell-specific α- and β-glucosidase activities found in II/*Bodo* compared to other experimental variants (48a). Under heavy protozoan grazing pressure, to grow at least as rapidly as the cells are grazed seems to be the most frequently occurring strategy of some bacterial strains within natural bacterioplankton assemblages dominated by small cells (27, 38). On the other hand, the II/*Urotricha* variant can exemplify a substrate-controlled system, in which no detectable shifts in bacterial community composition (with the taxonomic tools we used) were found.

The conditions of a continuous-flow system determine a certain rate of bacterial growth and therefore support the presence of bacterial populations that are physiologically active. This likely resulted also in the strongly enhanced cultivation efficiency (average, ~30% in all but the II/*Bodo* variant) compared to natural systems (9, 43). A change of physiological status of the majority of bacteria in the II/*Bodo* variant mediated through heavy grazing and consequently nutrient recycling could also have been responsible for the remarkably higher proportion of CFU (average, 88% of total bacteria). Moreover, some of these isolates readily grew after the reinoculation, while the strains belonging to the same species, at least according to the probability level, showed very limited culturability when isolated from other variants. In fact, a tight relationship between protistan grazing and the increased metabolic and physiological activities of bacterioplankton has been previously reported (24, 30, 41).

Both oligonucleotide probing and the cultivation-based approaches indicated, although differently, a strong shift in the community composition in the presence of the bacterivore (cf. Fig. 5 and Table 1). However, the output of both approaches cannot be appropriately linked. Significant cultivation-dependent shifts in bacterial community structure were demonstrated, even in much more nutrient-rich environments such as activated sludge (51). Organically enriched cultivation media favored the growth of organisms from γ-Proteobacteria but selected against β-Proteobacteria (51). In our system, the most frequently identified strain from various variants of the experiment was *A. lwoffii* (γ-Proteobacteria), which showed an extreme phenoplasticity as it was isolated from the six different types of colonies. However, the results of hybridization showed that γ-Proteobacteria were only important in the system at the end of the experiment. Also frequently isolated and identified strains of the genera *Flavobacterium* (groups 1 and 2, Table 1) would constitute, according to their proportion within the isolates, about 2 to 6% of total bacterial numbers. However, they usually accounted only for ~0.5% of cells detected with the CF oligonucleotide probe in all variants. This documents that cultivation-dependent techniques do not provide unbiased information compared to in situ hybridization with different group-specific rRNA targeted probes, giving, on average, a detectable fluorescent signal from >85% of the total DAPI bacterial counts in this study (23). It thus certainly yields less biased information about the actual composition of bacterial communities than the cultivation approaches.

We conclude that our cultivation system designed on the basis of a stable, low supply of a substrate commonly present in pelagic environments (12, 49) might be a powerful and reproducible instrument for testing responses of mixed bacterial assemblages to grazing impacts of different bacterivores. In the experiments, the bacterivorous flagellates induced fundamental physiological and community shifts in the bacterial assemblage, as follows: (i) the community shift from β- to α-Proteobacteria, (ii) the formation of grazing-resistant bacterial forms, and (iii) the very high cell-specific activity of bacteria postulated for the system controlled by grazing (cf. 53), as well as a shift in the physiological status of the community documented by an increased proportion of cultivable bacteria.

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