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Effect of protistan bacterivory on coastal bacterioplankton diversity

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ABSTRACT: Four protist exclusion experiments were conducted to test the hypothesis that marine bacterivorous protists selectively feed on different bacterioplankton genotypes and affect the taxonomic diversity of coastal marine bacterioplankton communities. In these experiments, the changes in bacterial community composition of seawater samples from which protists were removed by filtration were followed and compared to those of untreated control water samples. Bacterioplankton community structure was inferred from the relative abundance of bacterial small subunit rRNA genes (SSU rDNAs) by a recently developed technique (length heterogeneity analysis by PCR (LH-PCR; Suzuki et al. 1998). The results of the experiments show that the community structure did not dramatically change up to a 24 h incubation period in any of the treatments. However there were significant differences in filtered water samples and controls between 24 and 48 h of incubation. In the absence of bacterivores some SSU rDNAs that were rare in the original water samples dominated the bacterioplankton SSU rDNA pool after 48 h of incubation. Protists appeared to be capable of controlling bacterioplankton taxonomic diversity under these manipulated conditions. The results also agree with the hypothesis that aquatic bacterioplankton communities are composed of small cells that escape predation, and are in a state of low physiological turnover.

KEY WORDS: Bacterivory effects · Bacterioplankton diversity

INTRODUCTION

The relative importance of different mechanisms controlling bacterioplankton populations is a topic of debate in aquatic ecology. Several studies have attempted to estimate the relative importance of bottom-up versus top-down controls of bacterioplankton community structure, but the question remains unanswered (Shiah & Ducklow 1995, Dufour & Torréton 1996). Nonetheless, in the past decade, both theoretical and empirical evidence demonstrate that bacterivorous protists can select their prey based on size (Chrzanowski & Šimek 1990, Gonzalez et al. 1990, Monger & Landry 1992) or quality (Mitchell et al. 1988, Landry et al. 1991, Gonzalez et al. 1993) and can potentially control the size distribution of aquatic bacterioplankton. Sherr et al. (1992) have shown that bacterivorous protists selectively graze on dividing cells, therefore affecting bacterioplankton net production as well as standing stock. Furthermore, recent studies show that different size classes of freshwater bacterioplankton are differentially affected by bacterivory (Pernthaler et al. 1996) and that protist grazing rates are higher on active than on inactive bacterioplankton cells (del Giorgio et al. 1996). These results support the hypothesis that a large fraction of aquatic bacterioplankton is composed of small cells that escape predation, and that are in a state of low physiological turnover (Kjelleberg et al. 1987).

Assuming that bacterioplankton cells from different taxonomic groups represent different states of metabolic turnover, the same hypothesis suggests that bacterivorous protists could affect bacterioplankton genotypic community structure. Recent studies (Pernthaler et al. 1997, Šimek et al. 1997, Hahn & Höfle 1998) examined the effect of bacterivorous protists on the genotypic community composition of mixed assemblages of freshwater bacterioplankton growing in enriched continuous culture. These studies used oligonucleotide hybridization to bacterial small subunit

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(SSU) rRNA to assess community diversity and showed shifts in the community under grazing pressure. In these experiments the bacterial biomass became dominated by grazing-resistant cells belonging to the beta subdivision of *Proteobacteria*. Pernthaler et al. (1997) also reported that small, fast-growing cells belonging to the alpha subdvision of the *Proteobacteria* became the numerically dominant bacterioplankton type.

Here, I report indications that bacterivorous protists preferentially fed on bacterioplankton genotypes that were stimulated in bottle incubations of natural seawater samples and thus have the potential to affect the taxonomic diversity of natural bacterioplankton communities. To evaluate the effect of selective bacterivory by protists on marine bacterioplankton genotypic community structure, I followed the changes in the bacterioplankton community structure in 4 protist exclusion experiments. In these experiments the changes in coastal bacterioplankton communities that had protists removed by gentle filtration was compared to the changes in the bacterioplankton community of untreated controls. Bacterioplankton community structure was inferred using length heterogeneity analysis by PCR (LH-PCR), a recently developed PCR-based method that estimates the relative proportions of SSU rDNAs belonging to different bacterioplankton genotypes from their proportions in PCR products (Suzuki et al. 1998).

MATERIAL AND METHODS

Four protist exclusion experiments were performed using 4 different water samples from the Oregon coast. In these experiments bacteria were separated from bacterivorous protists by size fractionation. The community structure inferred by LH-PCR was followed with time and compared with that of an unfiltered control. Cell counts were performed for all samples, except when otherwise noted. The experiments are referred to hereafter as PROTEX 1 to 4.

Water samples. Water samples were collected at different times and locations off the Oregon coast. The water samples used for PROTEX 1 (March 28, 1993) and PROTEX 4 (July 24, 1997) were collected at subsurface (10 m) by Niskin (General Oceanics, Miami, FL) bottles (PROTEX 1) or from the surface using a bucket (PROTEX 4), aboard the RV 'Sacajawea' at a station located 8 km off the mouth of Yaquina Bay, Oregon (44° 39.1' N, 124° 10.6' W). The water sample used for PROTEX 2 (October 3, 1993) was collected at subsurface (10 m) by Niskin bottles aboard the RV 'Wecoma' at a station located off the mouth of the Columbia River, Oregon (47° 17.06' N, 124° 44.8' W). The water sample used for PROTEX 3 (March 25, 1997) was collected from the surface with a bucket at the South Jetty of the Yaquina Bay, Oregon (44° 36.8' N, 124° 10.6' W).

PROTEX 1. The water sample was pre-screened through a 10 µm mesh. Two 4 l subsamples were filtered through 0.8 µm polycarbonate membranes (Poretics, Osmonics, Minnetonka, MN) and used to fill two 4 l polycarbonate bottles. The controls consisted of two 4 l polycarbonate bottles filled with unfiltered water. At time zero and 24 h after the start of incubation in the dark at 15°C, the water from one of the treatment bottles and one of the control bottles was filtered through 0.2 µm polysulfone filters (Supor-200, Gelman Inc., Ann Arbor, MI). The filters were immersed in sucrose lysis buffer (40 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris·HCl pH 9.0) and stored at −80°C for later analysis.

PROTEX 2. Nine 4 l subsamples were filled with subsample filtered through a 2.0 µm polycarbonate membrane. Nine 4 l polycarbonate bottles were filled with unfiltered water and served as controls. At time zero and after 12 and 24 h incubation at surface temperature in an ondeck Plexiglas incubator the water from 3 of the treatment bottles and 3 of the control bottles was filtered through 0.2 µm polysulfone filters and the filters stored as above.

PROTEX 3 and 4. Seven 500 ml subsamples were filtered once through GF/F glass fiber filters (Whatman, Kent, UK) and twice through 0.8 µm polycarbonate membranes. To avoid the effects caused by separation of bacteria from bacterivorous protists and eukaryotic phytoplankton on substrate availability and nutrient feedback to the bacterioplankton, the samples were incubated in 500 ml polysulfone filter holders with receivers (Nalgene, Rochester, NY, Item 300-4050). The unfiltered controls were poured into the bottom collection flask, the filtered subsamples were gently poured in the top filtration tower closed with a lid and the subsamples were separated by a 0.2 µm polycarbonate membrane that allowed inorganic nutrients and organic substrates to exchange. At time zero and after 16, 24, 30, 37, 42 and 48 h (PROTEX 3) or 14, 24, 30, 34, 42 and 48 h (PROTEX 4) of incubation in the dark at 15°C under gentle shaking (the filtration flasks were lying on their sides), the water from one of the treatment bottles and one of the controls was filtered through 0.2 µm polysulfone filters and the filters stored as above.

Cell counts. In PROTEX 2, 20 ml samples were collected from all treatment and control subsamples, preserved, stained, and filtered onto 0.2 or 0.8 µm polycarbonate filters for enumeration of bacteria and of heterotrophic flagellates via DAPI staining (Sherr et al. 1993, Turley 1993). Bacterivorous protists were not enu-

merated in the controls of PROTEX 1. In PROTEX 1, 3 and 4, 20 ml samples were collected from 1 treatment and 1 control bottle at each time immediately before the remainder of the samples were filtered for nucleic acid samples. The sample for initial bacterial counts for the filtered sample of PROTEX 4 was accidentally lost.

LH-PCR. Total cellular nucleic acids were extracted from the polysulfone filters by lysis with proteinase K and SDS, followed by phenol/chloroform extraction as previously described (Giovannoni et al. 1990b). 10 ng of purified genomic DNA were used as the template for LH-PCR. Details of the LH-PCR protocol are described elsewhere (Suzuki et al. 1998). Briefly, in a final volume of 100 µl, reactions contained 0.2 mM of premixed dNTPs (Stratagene, La Jolla, CA), 1.5 mM $MgCl_2$, 5% acetamide and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI). The forward primer, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') (Giovannoni 1991), targeted SSU rDNAs from the domain Bacteria and was 5'-end-labeled with the phosphoramidite dye 6-FAM and purchased from Genset (San Diego, CA). The reverse primer, 355R (5'-GCT GCC TCC CGT AGG AGT-3') (Amann et al. 1990), also targeted SSU rDNAs from the domain Bacteria and was synthesized at the Central Services Laboratory, Center for Gene Research and Biotechnology, Oregon State University. All reactions used the Ampliwax™ hotstart protocol (Perkin Elmer-Cetus, Norwalk, CT) in a PLT100 thermal cycler (MJ Research Inc., Watertown, MA) programmed to 16 cycles of 96°C denaturation, 1 min, 55°C annealing, 1 min, and 72°C extension, 3 min.

The concentration of labeled PCR products was estimated after electrophoresis in an agarose minigel stained with ethidium bromide (0.5 µg ml^{-1}) and comparison with mass standards. The PCR products were purified using Qiaquick-spin columns (Qiagen, Chatsworth, CA) and approximately 10 fmol of the LH-PCR products were discriminated by Long Ranger (FMC, Rockland, ME) polyacrylamide gel electrophoresis in an Applied Biosystems Inc. 377 automated DNA sequencer (Applied Biosystems Inc., Foster City, CA) using the software Genescan® (Applied Biosystems Inc.). The software outputs electropherograms in which the bands are represented by peaks and the integrated fluorescence of each band is the area under the peaks. The relative abundance of each amplicon was estimated as the ratio between the integrated fluorescence of each of the peaks and the total integrated fluorescence of all peaks.

RESULTS

Cell counts

In all experiments the total numbers of bacteria in the filtered water treatments and in the controls increased with time (Table 1, Fig. 1), and except for PROTEX 4 (Fig. 1B), there was no decline in bacterial numbers in the control populations. In all experiments using water filtered through 0.8 µm, the initial num-

Table 1. Time series of bacteria and bacterivorous protists in experimental (filtered) and control (unfiltered) water samples. Cell numbers of PROTEX 2 are averages and standard deviations for tripicate bottles. bd: below detection, ns: no sample

Time (h)	PROTEX 1 Bacteria (10^6 cells ml^{-1})		PROTEX 2 Bacteria (10^6 cells ml^{-1})		PROTEX 2 Protists (10^2 cells ml^{-1})	
	<0.8 m	Unfiltered	<2.0 m	Unfiltered	<2.0 m	Unfiltered
0	1.67	2.07	2.21 + 0.15	2.14 0.12	1.51 1.33	8.42 3.79
12			2.86 0.07	2.29 0.11	0.85 0.58	10.4 2.87
24	2.90	2.37	3.34 0.14	1.65 0.20	3.37 1.54	8.59 3.64

Time (h)	PROTEX 3 Bacteria (10^6 cells ml^{-1})		PROTEX 3 Protists (10^3 cells ml^{-1})	PROTEX 4 Bacteria (10^6 cells ml^{-1})		PROTEX 4 Protists (10^3 cells ml^{-1})
	<0.8 m	Unfiltered	Unfiltered	<0.8 m	Unfiltered	Unfiltered
0	0.40	0.79	bd	ns	1.18	1.33
14	0.41	0.97	bd			
16				0.09	3.30	1.33
24	0.28	1.25	0.30	0.12	3.69	2.84
30	0.68	2.43	0.36	0.23	3.66	3.69
34				0.35	2.19	5.72
37	0.82	2.39	0.42			
42	1.24	3.10	0.73	1.53	2.56	6.42
48	2.03	4.49	1.03	3.01	1.17	5.03

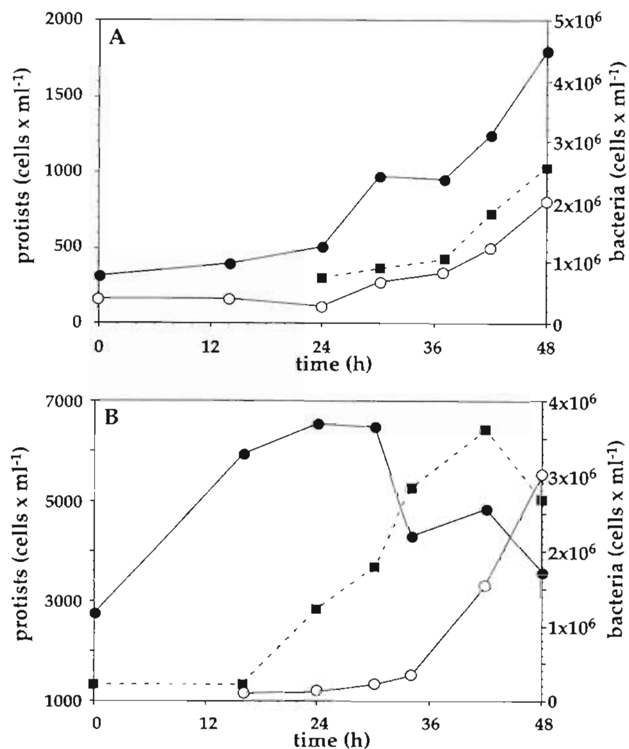

Fig. 1. Time course of bacteria and bacterivorous protists. (A) PROTEX 3. (B) PROTEX 4. (o) Bacteria, 0.8 µm filtered water; (●) bacteria, controls; (■) bacterivorous protists, controls

bers of bacteria in the treatment subsamples were lower and showed a longer lag phase than the controls.

Bacterivorous protist numbers increased with time after a lag phase in the controls of PROTEX 3 and 4 (Fig. 1) and did not grow in the 0.8 µm filtered samples. In PROTEX 4 bacterivores apparently controlled the increase in bacterial numbers since the increase in bacterivores was followed by a decrease in the total numbers of bacteria. Bacterivores did not show increasing or decreasing trends in the controls of PROTEX 2 (Table 1), but were present and increased in numbers in the 2.0 µm filtered water treatments, with final numbers reaching about 40% of the numbers in the controls (Table 1).

LH-PCR

Except for PROTEX 4, the initial community structure was very similar in all the samples (Table 2, Figs. 2A & 3A). The fragments sized between 315 and 317 base pairs (bp) (Peak 1) were the most abundant fragments in the original samples, representing up to 50% of the fluorescence of all amplified fragments. An analysis of the sizes of the region between primers 27F and 355R of 366 SSU rDNA sequences directly cloned from DNA samples from seawater or belonging to bacteria isolated from seawater showed that these frag-

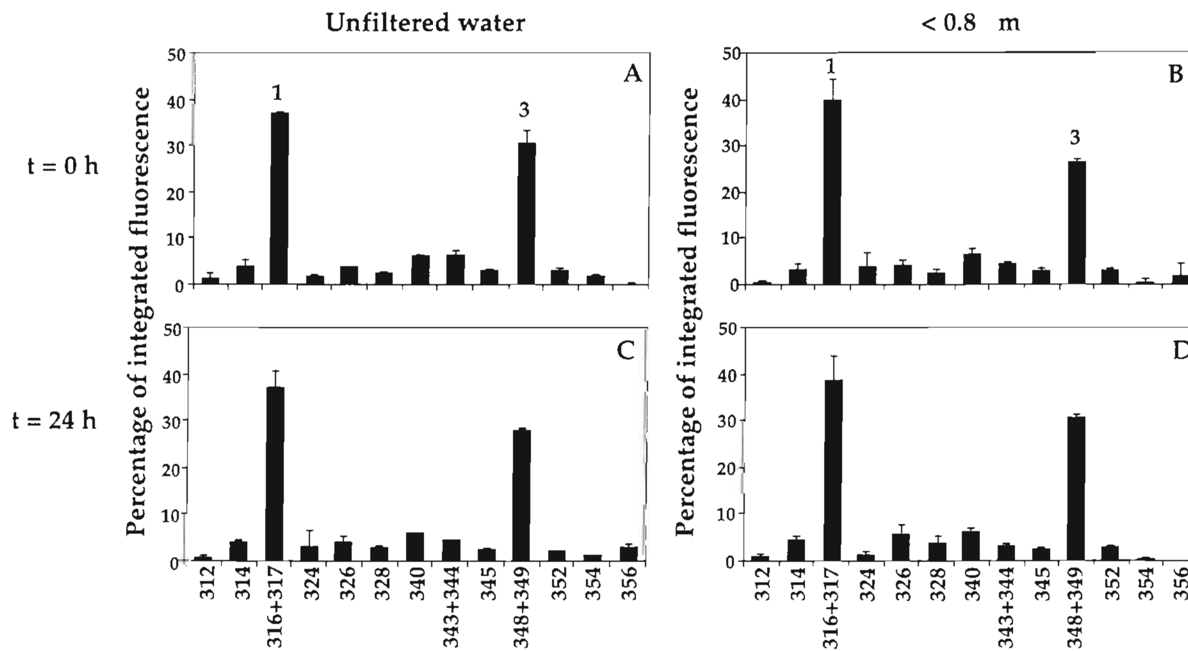

Fig. 2. PROTEX 2. Percentage of integrated fluorescence emission by rRNA of different lengths produced by PCR using primers 27F (5'-labeled with 6-FAM) and 355R. The *x*-axis represents the size of domains in base pairs, estimated by comparison to the size standard GS2500 (Applied Biosystems Inc.). Error bars are 1 standard deviation from the average between duplicate (unfiltered water) and triplicate bottles. (A) Control bottles, $t = 0$ h. (B) Treatment bottles $t = 0$ h. (C) Control bottles $t = 24$ h. (D) Treatment bottles, $t = 24$ h

Table 2. Time series of the ratio (%) of integrated fluorescence of PCR amplified SSU rDNAs of different sizes and the total integrated fluorescence of all PCR amplifed fragments assigned to bacterial SSU rDNAs. Percentages of PROTEX 2 are averages and standard deviation for duplicate (unfiltered water) and triplicate (<0.2 µm) bottles

PROTEX 1	Time	Fragment size (bp)										
28 March 1993	(h)	312	314	316+317	340	343	346	348	349	352+353	354	355
Unfiltered	0	3.1	1.7	57.6	3.7	4.8	0.0	10.1	8.9	1.7	8.4	0.0
	24	2.2	1.9	42.7	1.9	6.3	2.0	21.1	12.8	3.4	3.0	1.1
Filtered	0	3.9	2.5	50.3	2.4	6.5	0.0	11.5	8.1	2.4	12.4	0.0
(0.8 µm)	24	1.9	1.2	48.9	1.2	5.9	0.9	22.2	9.4	2.4	4.5	1.5

PROTEX 2		Fragment size (bp)												
3 October 1993		312	314	316+317	324	326	328	340	343+344	345	348 + 349	352	354	356
Unfiltered	0	1.3±1.3	3.8±1.1	36.8±0.1	1.5±0.1	3.8±0.1	2.6±0.3	6.1±0.2	6.1±1.3	3.0±0.2	30.6±2.8	2.8±0.3	1.5±0.3	0.2±0.2
	24	0.8±0.2	4.2±0.5	36.9±3.8	3.5±3.1	4.3±0.9	3.0±0.1	5.9±0.1	4.4±0.1	2.4±0.3	28.2±0.4	2.1±0.1	1.2±0.0	2.8±0.9
Filtered	0	0.5±0.2	3.5±0.9	39.7±4.7	3.7±3.3	4.3±1.1	2.6±0.5	6.4±1.4	4.4±0.4	2.9±0.3	26.6±0.6	3.0±0.5	0.6±0.6	1.8±2.6
(0.8 µm)	24	0.8±0.4	4.4±0.9	38.8±5.3	1.3±0.6	5.6±2.3	3.5±1.3	6.0±0.7	3.3±0.3	2.4±0.2	30.4±0.9	2.8±0.3	0.5±0.2	0.0±0.0

PROTEX 3		Fragment size (bp)											
25 March 1997		312	314	315–317	340	341	343+344	345	348	349+350	352	354	355
Unfiltered	0	1.0	0.0	48.3	3.3	0.0	10.3	3.8	18.7	10.6	2.6	1.4	0.0
	14	0.5	0.0	45.4	3.7	0.0	8.6	4.6	18.3	13.7	5.2	0.0	0.0
	24	0.0	0.0	30.6	2.5	0.0	2.0	4.6	30.8	16.4	13.1	0.0	0.0
	30	0.0	0.0	19.6	4.7	0.0	3.5	6.3	31.4	19.0	15.5	0.0	0.0
	37	0.0	0.0	23.5	6.4	0.0	3.8	6.6	31.2	17.2	11.3	0.0	0.0
	42	0.0	0.0	21.8	7.2	0.0	4.4	6.8	34.8	15.2	9.8	0.0	0.0
	48	0.0	0.0	23.9	5.8	0.0	1.7	5.6	39.3	15.2	8.6	0.0	0.0
Filtered	0	1.0	0.8	44.1	2.5	0.0	12.1	5.2	15.0	15.0	1.6	1.6	1.0
(0.8 µm)	14	0.9	0.4	46.7	2.1	0.0	10.5	4.5	14.4	15.0	1.5	1.7	2.1
	24	0.5	0.3	29.7	0.8	2.3	7.0	9.1	26.0	16.6	6.3	1.3	0.0
	30	0.0	0.0	11.5	0.0	4.9	4.9	13.5	35.3	19.5	10.4	0.0	0.0
	37	0.0	0.0	7.4	0.0	8.0	3.5	12.8	29.4	18.6	20.3	0.0	0.0
	42	0.0	0.0	2.6	0.0	23.2	2.2	2.9	19.3	12.1	37.7	0.0	0.0
	48	0.0	0.0	2.6	0.0	18.4	2.7	2.8	14.1	10.2	49.3	0.0	0.0

PROTEX 4		Fragment size (bp)											
24 July 1997		312	314	316+317	340	341	343+344	346	348	349+350	352	354	355
Unfiltered	0	0.0	1.1	24.8	0.0	5.2	12.0	5.2	36.7	8.1	5.2	1.8	0.0
	16	0.0	0.6	31.0	0.0	4.4	3.9	3.5	47.8	7.1	1.7	0.0	0.0
	24	0.0	0.0	25.0	0.0	4.7	5.0	4.2	49.6	7.0	4.7	0.0	0.0
	30	0.0	0.8	24.7	0.0	6.9	6.5	4.6	45.4	7.7	3.4	0.0	0.0
	34	0.0	1.3	26.5	0.0	5.0	5.2	4.6	43.7	7.2	6.6	0.0	0.0
	42	0.0	0.9	26.0	0.0	7.6	4.6	3.3	45.8	9.6	2.2	0.0	0.0
	48	0.0	0.0	20.6	0.0	6.1	5.1	3.7	47.1	8.7	8.0	0.0	0.0
Filtered	0	0.8	0.0	42.0	0.0	1.7	18.4	3.3	18.2	11.3	0.0	3.0	1.2
(0.8 µm)	16	0.5	0.0	39.2	0.0	3.8	19.3	3.7	19.3	10.7	0.0	3.4	0.0
	24	0.4	0.0	38.5	0.0	7.9	12.9	3.1	21.1	9.8	2.9	3.4	0.0
	30	0.0	0.0	7.9	0.0	33.6	8.3	0.0	25.8	7.3	14.9	0.0	2.3
	34	0.0	0.0	0.4	1.4	37.9	4.8	2.0	25.9	9.8	17.7	0.0	0.0
	42	0.0	0.0	0.7	3.5	75.4	0.0	0.0	7.9	0.0	12.6	0.0	0.0
	48	0.0	0.0	0.7	1.5	62.5	0.0	0.0	12.5	0.0	22.8	0.0	0.0

ment sizes correspond exclusively (except for Prymnesiophyte plastids) to the SSU rDNAs sequences of the alpha subdivision of the *Proteobacteria* (Suzuki et al. 1998). Furthermore, most of these sequences in the databases represent SSU rDNAs directly cloned from environmental DNA samples (Suzuki et al. 1998). The fact that the relative abundance of fragments sized between 315 and 317 bp did not show a noteworthy decrease after filtration through 0.8 µm strongly suggests that these fragments were of bacterial origin. Fragments sized 348 and 349 bp (Peak 3) were the second most abundant, representing up to 20% of the fluorescence of all amplified fragments. The size analysis of SSU rDNAs showed that these fragment sizes are shared by different phylogenetic groups, including the gamma and delta subdivisions of the *Proteobacteria*, and the *Flexibacter-Bacteroides-Cytophaga* phylum. Most of the sequences correspond to those of culti-

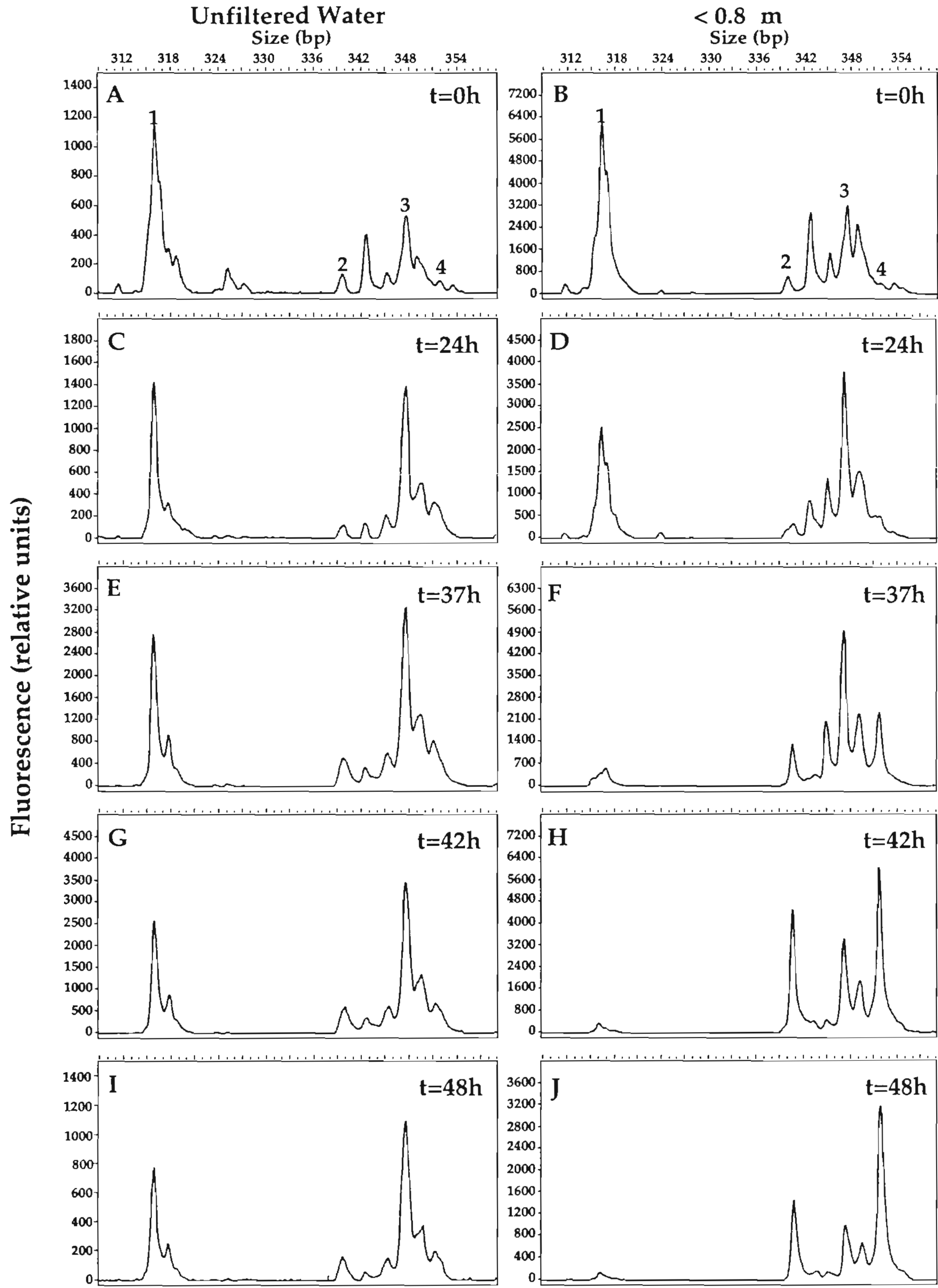

Fig. 3. PROTEX 3. rDNA amplified by PCR using primers 27F (5'-labeled with 6-FAM) and 355R. *x*-axis: size of fragments in base pairs estimated by comparison to the size standard GS2500 (Applied Biosystems Inc.). *y*-axis: relative fluorescence units. (A, C, E, G, I) control bottles; (B, D, F, H, J) treatment bottles

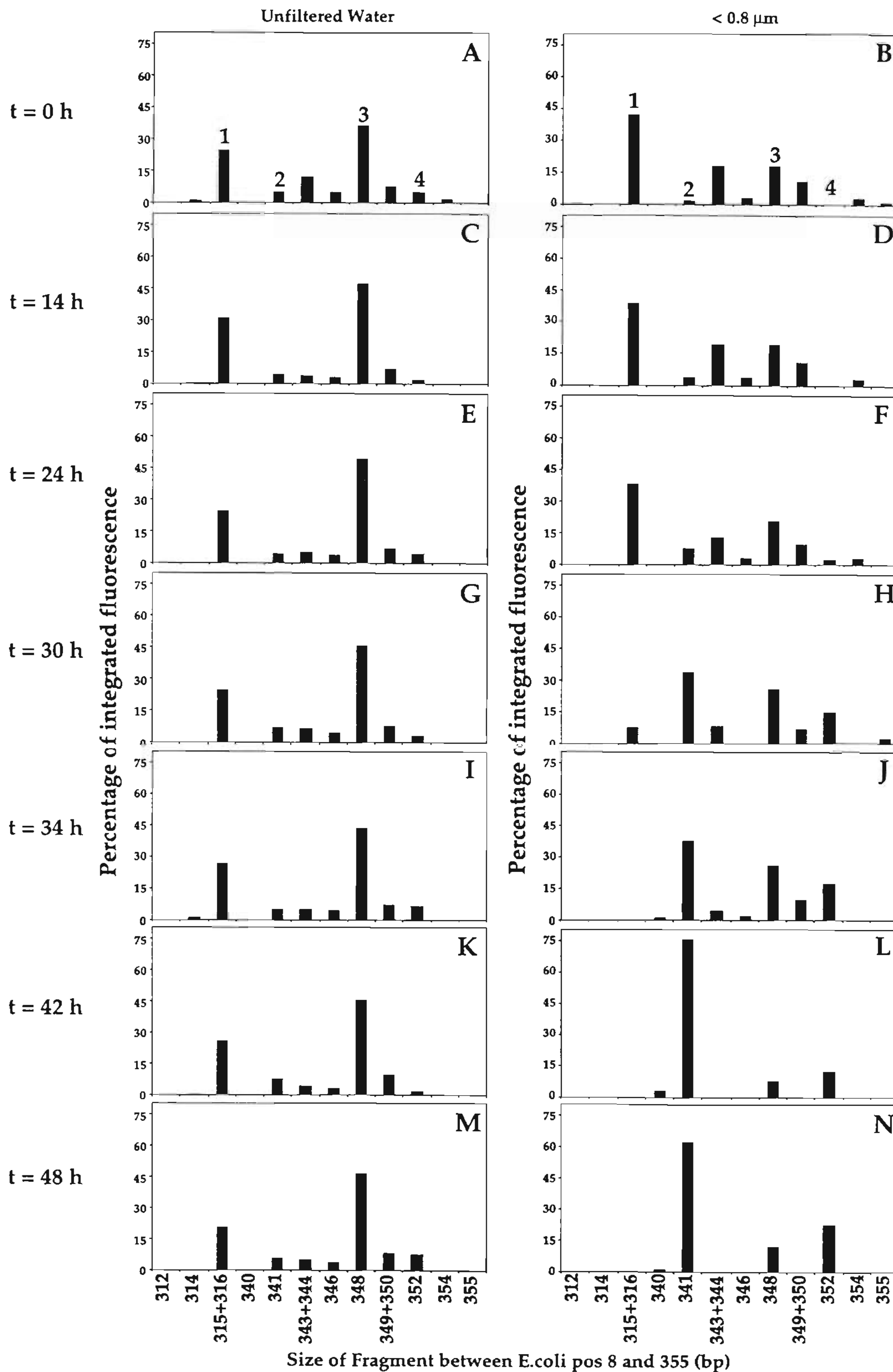

Fig. 4. PROTEX 4. Percentage of integrated fluorescence emission by different sized rDNAs amplified by PCR using primers 27F (5'-labeled with 6-FAM) and 355R. *x*-axis: size of fragments in base pairs estimated by comparison to the size standard GS2500 (Applied Biosystems Inc.). *y*-axis: relative fluorescence units. (A, C, E, G, I, K, M) control bottles; (B, D, F, H, J, L, N) treatment bottles

vated organisms (e.g. *Flexibacter littoralis*, *Oceanospirillum* spp., *Shewanella* spp., Suzuki et al. 1998).

Filtration through 0.8 or 2.0 µm did not dramatically change the community structure of the bacterioplankton inferred by LH-PCR (Table 2, Figs. 2A,B & 3A,B) in PROTEX 1 to 3, even though the initial bacterial counts in the filtered sample of PROTEX 3 was about 50% of the counts of the controls. This suggests that most bacterioplankton cells were smaller than 0.8 µm and/or that a similar fraction of organisms with SSU rRNA contributing to different peaks was smaller than 0.8 µm.

The community structure of the water sample used in PROTEX 4, inferred by LH-PCR, was different from that of other water samples (Fig. 4). The fragment sized 348 bp represented the largest fraction of total fluorescence (~40%) followed by fragments sized 315 and 316 bp (~25%). This difference might be attributed to the samples being collected during a phytoplankton bloom (pers. obs.). Furthermore, filtration through 0.8 µm changed the initial community structure, with an increase of the relative proportion of Peak 1, and a decrease in the proportion of Peak 3. This observation suggests that the bacteria contributing to the Peak 3 in PROTEX 4 were either larger and/or particle-associated. Also, although initial numbers of bacteria were not estimated for the unfiltered sample, the bacterial numbers of the 16 h sample indicate that there was a large difference in initial numbers between filtered and unfiltered samples.

The time courses of community structure showed certain patterns common to almost all of the experiments (Table 2, Figs. 2 to 4). (1) Except for PROTEX 2, the relative fluorescence of Peak 1 decreased with time in all filtered samples as well as in all the controls, while the relative fluorescence of Peak 3 increased with time. (2) After the first 24 h of incubation the community structure did not change in the filtered water treatments or controls to the same extent as the changes in community structure in treatments and controls incubated for 48 h. (3) After the first 24 h of incubation in PROTEX 1 to 3 there was no prominent difference between the changes in community structure in the filtered treatments and in controls.

Between 24 and 48 h of incubation (Table 2, Figs. 3E to I & 4G to N) there were remarkable differences in the patterns of change in genotypic community structure between filtered samples and unfiltered controls in PROTEX 3 and 4, suggesting that protists were selectively grazing specific bacterial genotypes. In both experiments, the community structure of the 0.8 µm filtered samples showed major shifts in the proportions of peaks between 24 and 48 h, while these changes were less pronounced in the controls. In PROTEX 3, the relative fluorescence of Peak 1 decreased several fold, while Peak 3, followed by several other peaks, dominated the population (Fig. 3F to J). After 42 h of incubation fragments sized 341 bp (Peak 2) and 352 bp (Peak 4) increased to about 25 and 40% of the total fluorescence of the 0.8 µm filtered samples, and after 48 h incubation, Peak 4 represented about 50% of the total fluorescence. The decrease in dominance of Peak 1 was less pronounced as it represented about 20% of the total fluorescence after 48 h in the controls. In the 0.8 µm filtered samples of PROTEX 4, Peak 2 represented about 30% of the total fluorescence after 30 h, and after 48 h became the sole dominant peak (~65% of the total fluorescence). In contrast, only a slight decrease in the percentage of fluorescence of Peak 1 was observed after 48 h of incubation of control samples.

The size analysis of the SSU rDNAs indicates that a fragment of 341 bp could represent different phylogenetic groups, including the beta and gamma subdivisions of the *Proteobacteria*, and the *Flexibacter-Bacteroides-Cytophaga* phylum, most of which are cultivated species (e.g. *Pseudoalteromonas* spp., *Flavobacterium* spp.) (Suzuki et al. 1998). The fragment of 352 bp represents marine strains and gene clones of the gamma (environmental clone SAR145, *Shewanella hanedai*) and beta (*Nitrococcus mobilis*) subdivisions of the *Proteobacteria* (Suzuki et al. 1998).

DISCUSSION

Selective feeding by aquatic bacterivorous protists has been shown empirically, (Chrzanowski & Šimek 1990, Gonzalez et al. 1990, 1993, Landry et al. 1991, Monger & Landry 1992, Sherr et al. 1992, del Giorgio et al. 1996, Pernthaler et al. 1996) and has been corroborated by physical models (Monger & Landry 1990, 1991, Gonzalez 1996). Earlier studies showed that bacterivores selectively grazed on larger bacterial analogs (Chrzanowski & Šimek 1990, Gonzalez et al. 1990). Later studies showed also preferential grazing rates on live bacterial cells rather than on heat-killed bacteria. However, the fact that this preferential feeding was only significant for motile bacteria (Monger & Landry 1992, Gonzalez et al. 1993) led to the suggestion that protistan selective feeding is a function of encounter rates between bacterivores and bacteria, and not a behavioral response by the protists.

Recent studies reinforced the importance of protists' selective feeding to the dynamics of microbial food webs. Observations that protists selectively feed on dividing bacterial cells, led to the hypothesis that protists not only crop bacterial standing stock, but more importantly, selectively feed on the bacterioplankton responsible for secondary production (Sherr et al.

1992). This hypothesis was supported by results of a study analyzing bacterioplankton size structure in a freshwater lake (Pernthaler et al. 1996). This study showed that bacterivores affected the most active bacterial size class, while abundant, small sized and relatively inactive bacteria were weakly affected by protistan bacterivory (Pernthaler et al. 1996). Finally, the recent report of selective bacterivory on active cells (i.e. capable of reducing the tetrazolium salt CTC; del Giorgio et al. 1996) further supports the hypothesis that bacterivorous protists not only affect bacterioplankton standing stock, but also bacterioplankton net production.

Analysis of replicated bottles for PROTEX 2 showed that the community structure inferred by LH-PCR was consistent between bottles, with a coefficient of variation in general lower than 20% for most peaks, and patterns of dominance conserved between bottles. Furthermore, the consistency between sequential samples collected in sequential time in PROTEX 3 and 4 strongly suggests that these patterns were a result of changes in the community structure inferred by LH-PCR, rather than random experimental error or PCR bias. As in the original description of the method, LH-PCR analysis did not consistently discriminate peaks of adjacent fragments, and therefore, the fluorescence of some adjacent fragments were combined (i.e. fragments of 348 and 349 bp). Although this represents a complicating factor in the interpretation of the results, peaks of fragments with similar sizes, in general, represent the same phylogenetic groups (Suzuki et al. 1998).

The results of PROTEX 3 and PROTEX 4 suggest that protists preferentially fed on the fraction of the bacterioplankton growing in the experimental bottles. In both experiments, the structure of the bacterioplankton community changed with time and proportion of the community representing the larger fraction of SSU rDNAs in the original water samples decreased in treatments where protists were absent, as well as in the controls. The percentage of SSU rDNAs of bacteria composing Peaks 2, 3 and 4 dramatically increased while the percentage of SSU rDNAs of bacteria representing the dominant peak in the original samples (Peak 1) decreased after 48 h. In contrast, in controls with protists the percentage of SSU rDNAs corresponding to Peaks 2, 3, and 4 increased to a lesser extent.

The most parsimonious explanation for the increase in proportions of Peaks 2, 3 and 4 in the absence of protists is that the bacteria contributing to those peaks had higher growth rates than those contributing to Peak 1. Regardless of the differences in operon copy numbers between different organisms, if the growth rates had been the same, the proportions between the peaks would not have changed. Higher growth rates of bacteria representing Peaks 2, 3 and 4, combined with preferential bacterivory by protists, could also explain the smaller increase in the proportion of these peaks in the controls.

The fact that the initial bacterial counts were lower in filtered samples than in the controls of PROTEX 3 and 4 represents a complicating factor to the interpretation of the results. It is possible that the filtration step could have considerably modified the genotypic diversity and affected the distribution of size, biomass and activity between different members of the community. There is little evidence that the bacteria contributing to Peaks 2, 3 and 4 were larger than those contributing to Peak 1, in the original water samples, since except for PROTEX 4, the relative proportion between the peaks in 0.8 µm filtered samples at time zero was similar to that of the unfiltered samples. Also, the fact that the most pronounced change in community structure in PROTEX 3—namely, the decrease in proportion of Peak 1—was the same in filtered samples and unfiltered controls suggests that filtration alone could not explain the results of PROTEX 3. However, filtration definitely had an effect in PROTEX 4, since the community structure at time zero was different between filtered samples and controls.

Higher mortality rates of bacteria representing Peak 1 in the absence of protists could be also invoked to explain the results. Viral lysis is such an alternative source of bacterial mortality. However, mortality by viral lysis was likely lowered in the filtered samples since encounter rates between viral particles and their hosts was decreased by the lower numbers of bacteria. Furthermore, Peak 1 in LH-PCR represents genes belonging to the alpha subdivision of the *Proteobacteria*. In most marine samples studied to date this subdivision is represented by organisms belonging to several groups (Suzuki & DeLong in press). Therefore it is very unlikely that bacteria representing Peak 1 should be entirely and uniquely affected by bacteriophage infection and lysis.

Alternatively, if the community was affected by viral lysis in the original water sample, the decrease in encounter rates between bacteriophages and bacteria could have released some bacteria from this mortality source in filtered water samples, especially in PROTEX 4. However, the observations that the peak that became dominant in PROTEX 4 (Peak 2) represented a small fraction of the peaks in the original population, as well as that this peak continuously dominated the population from 30 to 48 h, does not support the release from viral mortality as a likely explanation to the differences in the changes in community structure between filtered and unfiltered samples.

Another explanation to the results could be lower availability of substrates (from decaying phytoplankton) and inorganic nutrients (regenerated by the protists) in the 0.8 µm filtered treatments, and the possibil-

ity that organisms contributing to Peaks 2, 3 and 4 are better adapted to conditions of substrate and nutrient limitation. However, it is likely that some cell breakage and release of nutrients and substrates occurred during filtration. Added to this, the fact that there was a certain degree of exchange of nutrients and substrates between the chambers precludes the assumption that there were major differences in nutrient and substrate loads between the chambers. The fact that the maximum net growth rates of bacteria in filtered samples in PROTEX 3 and 4 were higher than the maximum net growth rates in the controls—observed before the increase in protist numbers—does not indicate nutrient or substrate limitation in filtered samples, at least early in the incubations. However, the observations that net community growth rates in filtered samples decreased towards the end of the incubations, as well as that the integrated growth rates between 0 and 48 h in PROTEX 3 were similar between filtered and unfiltered samples, indicate that substrate or nutrient limitation may contribute to the changes observed after 36 h in PROTEX 3 and after 42 h in PROTEX 4.

Finally, the long lag times before growth in 0.8 µm filtered samples suggest that the bacteria contributing to Peaks 2, 3 and 4 were not active in the original water sample and were stimulated by sample manipulation. Ferguson et al. (1984) showed that the number of bacteria capable of forming colonies in enriched agar plates increased after sample confinement and manipulation. The fact that most SSU rRNA sequences of bacteria cultivated or cloned from seawater, and with sizes corresponding to Peaks 2, 3 and 4 in sequence databases, are indeed those of previously cultivated bacteria agrees with the suggestion that colony-forming bacteria are stimulated by manipulation.

Although the results showed that bacterivorous protists have the potential to preferentially graze on bacteria stimulated in manipulation experiments, it is not possible to directly extrapolate the results to *in situ* microbial communities. Analysis of the contents of protists' food vacuoles could be an alternative way to examine preferential feeding by bacterivores. Two recent studies have succeeded in hybridizing fluorescent oligonucleotides to cultivated bacteria inside bacterivorous protists' food vacuoles (Gunderson & Goss 1997, Pernthaler et al. 1997). However, the possibility that most *in situ* bacterioplankton communities are slow-growing cells that may not have enough ribosomes to allow detection presents a problem to the use of oligonucleotide hybridization to bacteria inside food vacuoles to estimate preferential grazing by protists.

The combined observations that: (1) all known SSU rRNA sequences of bacteria cultivated or cloned from seawater and with the sizes of Peak 1 in sequence databases represent sequences of alpha *Proteobacteria*; (2) Peak 1 was dominant in most of the original samples; (3) this peak represented organisms that were outgrown in seawater culture with no protists; and (4) the organisms representing this peak were apparently grazed to a lesser extent than other bacteria support the hypothesis that a large fraction of aquatic bacterioplankton is composed of small cells that escape predation, representing a state of low physiological turnover (Kjelleberg et al. 1987). This hypothesis also helps to explain the discrepancy between the sequences of SSU rDNA of bacterioplankton directly cloned from seawater to those of marine bacterioplankton obtained by cultivation methods (Giovannoni et al. 1990a, Schmidt et al. 1991, DeLong et al. 1993, Fuhrman et al. 1993, Suzuki et al. 1997), as well as recent observations that a large proportion of natural bacterioplankton communities are relatively inactive (del Giorgio & Scarborough 1995, Gasol et al. 1995, del Giorgio et al. 1997). The same observations above seem at first to contradict those by Pernthaler et al. (1997), since grazing on smaller alpha *Proteobacteria* did not appear to be lower than on other bacterial types in their continuous culture experiment. However, in that study the community shifted in the first 24 h of incubation from being dominated by larger (J. Pernthaler pers. comm.) beta *Proteobacteria* in the absence of flagellates to being dominated by smaller alpha *Proteobacteria* in the presence of flagellates, in agreement with the observations presented here. Differences between freshwater and marine bacterial assemblages, as well as the fact that the Pernthaler et al. (1997) assumed that grazing was density-dependent, might also explain the discrepancy between the results of the study of Pernthaler et al. and those presented here.

Since the results of the present study might be the result of bottle effects rather than reflect the dynamics of bacterioplankton populations *in situ*, the controversy regarding the phylogenetic diversity of active and inactive fractions of bacterioplankton populations (Rehnstam et al. 1993, Pinhassi et al. 1997, Suzuki et al. 1997) is far from resolved.

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