

## Effect of Protistan Grazing on the Frequency of Dividing Cells in Bacterioplankton Assemblages†

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Received 27 January 1992/Accepted 19 May 1992

Grazing by phagotrophic flagellates and ciliates is a major source of mortality for bacterioplankton in both marine and freshwater systems. Recent studies have demonstrated a positive relationship between clearance rate and prey size for bacterivorous protists. We tested the idea that, by selectively grazing the larger (more actively growing or dividing) cells in a bacterial assemblage, protists control bacterial standing stock abundances by directly cropping bacterial production. Samples of estuarine water were passed through 0.8- $\mu\text{m}$ -pore-size filters (bacteria only) or 20- $\mu\text{m}$ -mesh screens (bacteria and bacterivorous protists) and placed in dialysis tubing suspended in 7 liters of unfiltered water. Changes in total bacterial biovolume per milliliter (bacterial biomass), frequency of dividing cells (FDC), and average per cell biovolume were followed over a period of 24 h. In three experiments, the FDC increased more rapidly and attained higher values in water passed through 0.8- $\mu\text{m}$ -pore-size filters (average, 5.1 to 8.9%; maximum, 15.5%) compared with FDC values in water passed through 20- $\mu\text{m}$ -mesh screens (average, 2.7 to 5.3%; maximum, 6.7%). Increases in bacterial biomass per milliliter lagged behind increases in FDC by about 4 to 6 h. Grazed bacterial assemblages were characterized by lower total biomasses and smaller average cell sizes compared with those of cells in nongrazed assemblages. We conclude that bacterivorous protists control bacterial standing stock abundances partly by preferentially removing dividing cells. Selective grazing of the more actively growing cells may also explain, in part, the ability of slow-growing cells to persist in bacterioplankton assemblages.

Suspended bacteria in both marine and freshwater environments are characterized by relatively low standing stock abundances ( $10^5$  to  $10^7$  cells  $\text{ml}^{-1}$ ) and by small average per cell biovolumes (0.02 to 0.20  $\mu\text{m}^3$ ), compared with the high standing stock abundances ( $>10^8$  cells  $\text{ml}^{-1}$ ) and larger biovolumes ( $>0.5$   $\mu\text{m}^3$ ) typical of bacterial isolates grown in laboratory cultures (1, 8, 11). It is generally accepted that grazing by phagotrophic protists, including flagellates and ciliates  $<20$   $\mu\text{m}$  in size, is a major source of mortality for suspended bacteria, thereby keeping bacterial abundances low (4, 15). Several investigators have also proposed that a demonstrated positive relationship between bacterial cell size and bacterivore grazing rate results in more intense grazing pressure on larger bacteria than on smaller bacteria in the assemblage (2, 6, 8, 11-13). We previously hypothesized that, since the larger bacteria in a bacterial assemblage are generally the most actively growing and dividing cells, bacterivorous protists may be directly cropping bacterial production (as dividing cells) rather than simply randomly grazing the bacterioplankton postproduction standing stock (8).

Here we report the results of a test of this hypothesis. We determined the impact of protistan grazing on the frequency of dividing cells (FDC) in marine bacterioplankton assemblages. Selective grazing of dividing cells would not necessarily decrease overall bacterial biomass production; it would suggest a direct shunt of bacterial biomass increase to grazing protists rather than to new bacterial cells in the assemblage. We predicted that such an effect would result in FDC values that were lower in grazed natural bacterioplank-

ton assemblages than in ungrazed assemblages. We also determined the effect of grazing on the bacterial abundance (numbers and biovolume per milliliter) and on the average bacterial cell size.

### MATERIALS AND METHODS

Experiments I and II were set up in July 1989, and experiment III was set up in May 1990. Samples of estuarine water were collected in clean polycarbonate carboys at either slack high or slack low tide in a tidal creek adjacent to Sapelo Island, Ga., from a depth of 10 cm. Water samples of several hundred milliliters were gently screened through 20- $\mu\text{m}$ -mesh netting; 100-ml aliquots were filtered through 0.8- $\mu\text{m}$ -pore-size Nuclepore filters. Subsamples (50 ml) of the filtered (0.8- $\mu\text{m}$ -pore-size filters) and screened (20- $\mu\text{m}$ -mesh screens) water were gently poured into 40-cm lengths of cellulose dialysis membrane (Spectra/Por membrane, molecular weight cutoff of 50,000, 28-mm width; Spectrum Medical Industries, Los Angeles, Calif.). The dialysis membrane was boiled and copiously rinsed with deionized water before use. The filled lengths of membrane, clamped at both ends, were placed in an 8-liter polycarbonate carboy containing 7 liters of unscreened estuarine water. The experimental system was incubated at ambient temperature (30°C in July, 25°C in May); the water in the carboy containing the intact microbial community was gently stirred with a magnetic stirrer during the incubation to ensure constant diffusion into the dialysis bags of nutrients needed for bacterial growth.

Duplicate 400- $\mu\text{l}$  samples were taken from each of the two dialysis bags (containing filtered [0.8- $\mu\text{m}$ -pore-size filter] and screened [20- $\mu\text{m}$ -mesh screen] samples) 9 or 10 times over 24 h. The samples, preserved with 3% (final volume) borate-buffered Formalin, were stained with acridine orange and

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† Contribution no. 708 of the University of Georgia Marine Institute.

TABLE 1. Abundance of phagotrophic protists in the screened (20- $\mu\text{m}$ -mesh screen) samples at the beginning ( $T_0$ ) and end ( $T_{24}$ ) of the experiments

Expt	No. of protist cells per ml			
	$T_0$		$T_{24}$	
	Flagellates	Ciliates	Flagellates	Ciliates
I	2,810	206		
II	2,490	36	11,500	85
III	1,740	54	3,400	118

filtered onto 0.2- $\mu\text{m}$ -pore-size Nuclepore-black membrane filters for enumeration of bacteria (10). For each sample, 300 to 400 bacterial cells were counted at a magnification of  $\times 1,250$  and scored for the presence or absence of a visible division furrow to calculate the frequency of dividing cells (9). Fifty randomly selected bacterial cells were also measured with an ocular bar micrometer at a magnification of  $\times 2,500$  to determine the average cell biovolume in experiments I and II. For experiment III, the average bacterial cell size was determined by taking micrographs of 15 randomly selected fields at a magnification of  $\times 1,250$  with Kodak TMAX 400 film and then sizing from 200 to 300 cells in projected images of the frames taken for each individual sample. Total bacterial biovolume per milliliter was calculated as the product of the bacterial abundance and the average bacterial cell size for each sample.

To determine abundance of phagotrophic protists, at the beginning of the experiments 10-ml subsamples of filtered (0.8- $\mu\text{m}$ -pore-size filters) and screened (20- $\mu\text{m}$ -mesh screens) water were preserved with 2% (final volume) borate-buffered Formalin and stained with 4',6-diamidino-2-phenylindole by a modification of the Porter and Feig (16) method. A 100- $\mu\text{l}$  aliquot of a 4',6-diamidino-2-phenylindole (1 mg/ml) solution made with deionized water was injected into each 10-ml sample; after 7 to 10 min, the sample was filtered onto 0.8- $\mu\text{m}$ -pore-size Nuclepore-black membrane filters. For experiments II and III, protist samples were also collected at the end of the incubations. The numbers of nonpigmented flagellates and phagotrophic ciliates per milliliter were determined as described by Sherr et al. (20). All microscopy was done with a Zeiss Universal epifluorescence microscope outfitted with UV and blue light filter sets and an Optivar unit that allowed enlargement of bacterial cells by  $\times 2,000$ .

## RESULTS

The abundances of nonpigmented flagellates and ciliates were in the range of  $1.7 \times 10^3$  to  $2.8 \times 10^3/\text{ml}$  and 40 to 200/ml, respectively, in the screened (20- $\mu\text{m}$ -mesh screen) water at the start of each of the three experiments (Table 1). Both flagellates and ciliates increased in abundance during experiments II and III (Table 1). The flagellates were predominately monads 4 to 6  $\mu\text{m}$  in diameter, and the ciliates were scuticociliates and choreotrichs 10 to 20  $\mu\text{m}$  in diameter. During summer, the protistan assemblage in these tidal creek waters was previously shown to clear the water of bacterioplankton at an average rate of 2.7 nl per cell per h for flagellates and 168 nl per cell per h for <20- $\mu\text{m}$  ciliates (20). Combining these clearance rates with the cell abundances of both flagellates and ciliates measured at the beginning of the experiments, we estimated that the protistan assemblages in the samples passed through 20- $\mu\text{m}$ -mesh screens would have been capable of clearing 101, 30, and 33% of the water per

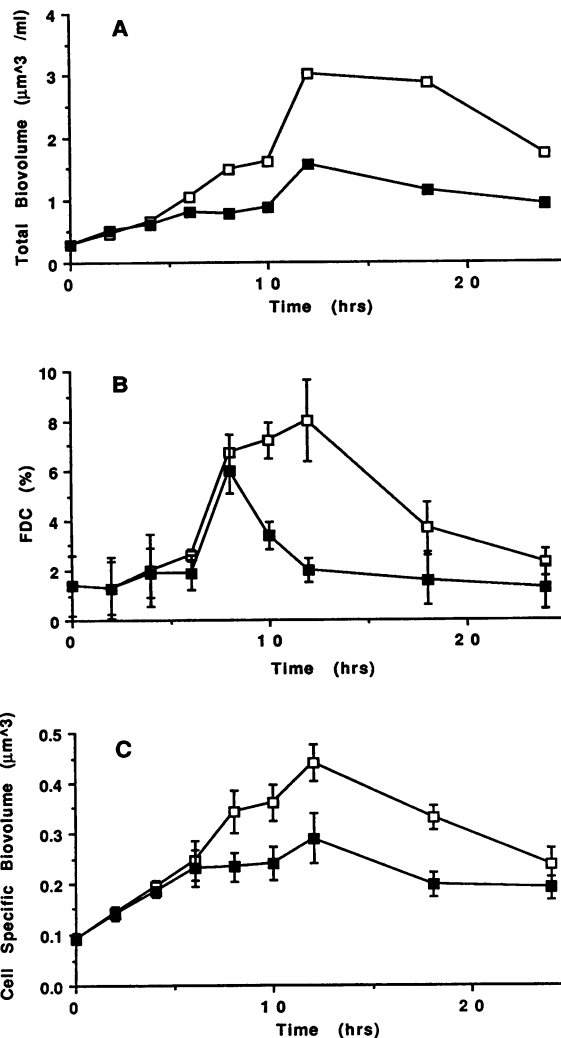


FIG. 1. Variation in bacterial standing stock biovolume ( $10^6 \mu\text{m}^3/\text{ml}$ ) (A), percent FDC (B), and average bacterial cell size (C) for bacteria only ( $\square$ ; water passed through 0.8- $\mu\text{m}$ -pore-size filters) and bacteria and protists ( $\blacksquare$ ; water passed through 20- $\mu\text{m}$ -mesh screens) during experiment I. Each error bar indicates 1 standard error.

day in experiments I, II, and III, respectively. Assuming the same grazing rates, by the end of experiments II and III the protist assemblages would have been clearing 109 and 70% of the water per day, respectively. No protists were observed in the water samples passed through 0.8- $\mu\text{m}$ -pore-size filters at the beginning or end of the experiments.

In the experiments, the three parameters of bacterial growth measured (total bacterial biovolume [numbers  $\times$  average cell size]/milliliter, FDC, and average per cell biovolume) began to increase after approximately 4 to 6 h (Fig. 1 through 3). In each experiment, the total increase in cell abundance and cell biovolume per milliliter from 6 to 24 h was greater in the filtered (0.8- $\mu\text{m}$ -pore-size filters) samples (bacteria only) than in the screened (20- $\mu\text{m}$ -mesh screens) samples (bacteria plus protists) (Table 2). In addition, the mean cell biovolume and the FDC for samples taken between 6 and 24 h were consistently greater in the filtered samples than in the screened samples (Table 2). The maximum FDC observed ranged from 7.2 to 15.5% with the

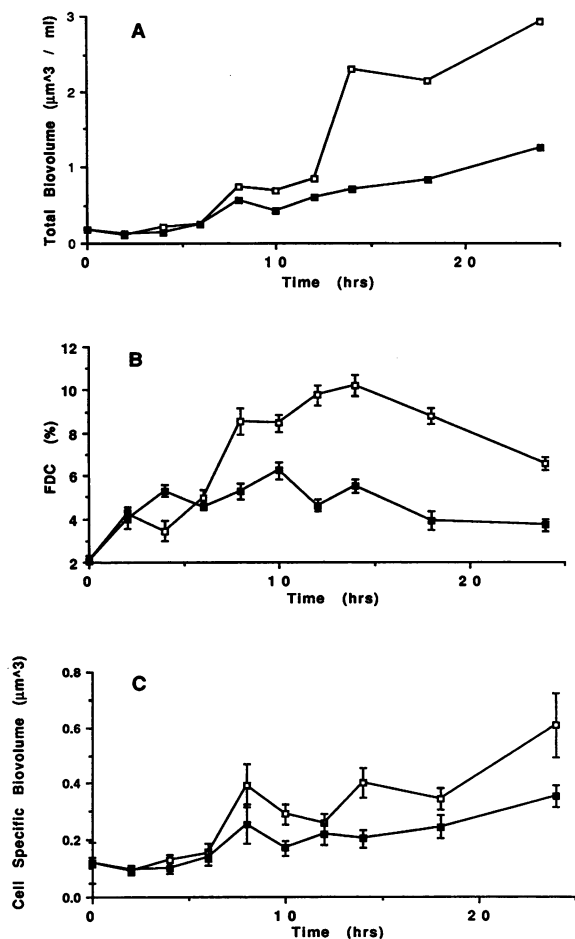


FIG. 2. Variation in bacterial standing stock biovolume ( $10^6 \mu\text{m}^3/\text{ml}$ ) (A), percent FDC (B), and average bacterial cell size (C) for bacteria only ( $\square$ ; water passed through 0.8- $\mu\text{m}$ -pore-size filters) and bacteria and protists ( $\blacksquare$ ; water passed through 20- $\mu\text{m}$ -mesh screens) during experiment II. Each error bar indicates 1 standard error.

nongrazed treatments and from 5.0 to 6.2% with the grazed treatments.

In each case, a marked increase in FDC preceded any increase in total bacterial biovolume per milliliter by about 6 h (Fig. 1 through 3). Although the FDC and the average cell biovolume also increased in the screened (20- $\mu\text{m}$ -mesh screens) samples, the values observed for these two parameters were consistently lower in samples with grazing than in samples with bacteria only. In addition, peaks in FDC were lower and of shorter duration in grazed treatments than in nongrazed treatments (Fig. 1 to 3).

In theory, FDC and average cell biovolume should be positively related. Dividing cells are larger than nondividing cells; thus, a bacterial assemblage with a higher proportion of dividing cells would be expected to have a greater mean cell biovolume. We tested this idea by comparing empirically determined average cell biovolumes and FDCs for individual samples from both treatments during the three experiments. The resulting relationship shows a positive, although not highly significant ( $r = 0.65$ ), slope (Fig. 4).

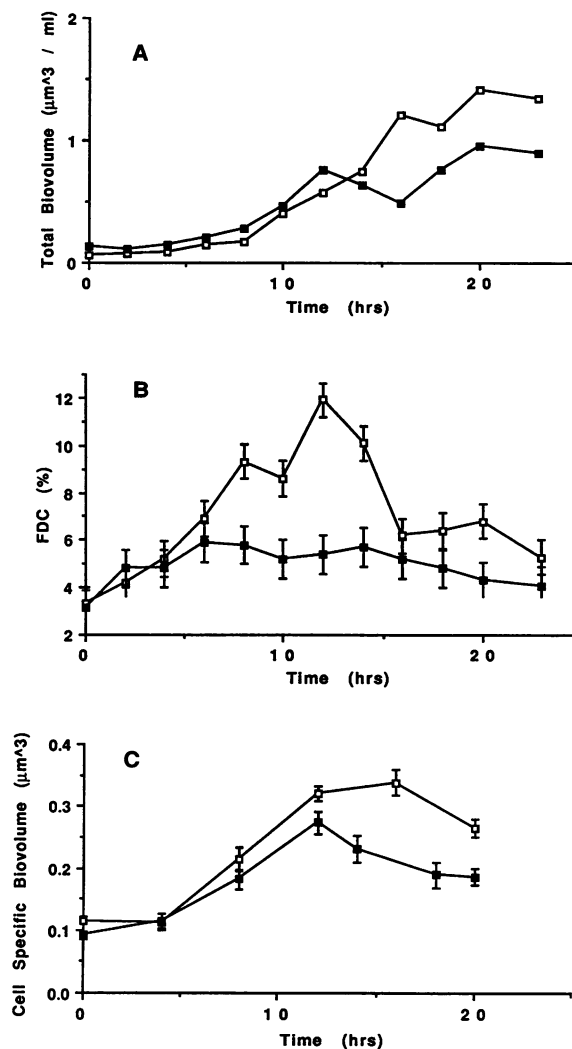


FIG. 3. Variation in bacterial standing stock biovolume ( $10^6 \mu\text{m}^3/\text{ml}$ ) (A), percent FDC (B), and average bacterial cell size (C) for bacteria only ( $\square$ ; water passed through 0.8- $\mu\text{m}$ -pore-size filters) and bacteria and protists ( $\blacksquare$ ; water passed through 20- $\mu\text{m}$ -mesh screens) during experiment III. Each error bar indicates 1 standard error.

## DISCUSSION

Prevailing theory suggests a tight coupling between microbial predators and prey in aquatic ecosystems (4, 7, 15). Bacterivorous protists, including flagellates and ciliates, have been shown to clear bacterium-sized prey from a large fraction of the water column each day in freshwater (11, 15, 22) and marine (15, 20) systems. It has further been suggested by Krambeck (11) and by Gonzalez et al. (8) that bacterivores are, in fact, cropping the production rather than simply the standing stock of suspended bacteria because of a higher grazing pressure on larger cells in the assemblage. The larger cells were assumed to be the ones that are most actively growing and to include most of the dividing cells (8).

Several experimental studies have corroborated the idea that bacterivorous protists graze the larger cells in bacterioplankton assemblages at rates severalfold higher than they do the smallest cells in the assemblages. With regard to changes in the cell size spectrum of grazed bacterial assemblages, Andersson et al. (2) reported that a heterotrophic

TABLE 2. Results for samples taken between 6 and 24 h during the experiments

Expt and filter treatment <sup>a</sup>	Increase in:		Avg cell biovolume, mean $\mu\text{m}^3$ (SD) <sup>b</sup>	FDC, mean % (SD) <sup>b</sup>
	Cell abundance ( $10^7$ cells/ml)	Biovolume ( $10^6 \mu\text{m}^3/\text{ml}$ )		
I				
0.8 $\mu\text{m}$	3.0	1.06	0.32 (0.08)	5.1 (2.3)
20 $\mu\text{m}$	1.5	0.32	0.23 (0.03)	2.7 (1.6)
II				
0.8 $\mu\text{m}$	3.2	2.67	0.35 (0.13)	8.2 (1.7)
20 $\mu\text{m}$	1.6	0.99	0.23 (0.06)	4.8 (0.8)
III				
0.8 $\mu\text{m}$	4.7	1.19	0.28 (0.05)	8.9 (2.9)
20 $\mu\text{m}$	3.4	0.69	0.20 (0.03)	5.3 (1.1)

<sup>a</sup> Samples were passed through 0.8- $\mu\text{m}$ -pore-size filters (0.8  $\mu\text{m}$ ) or 20- $\mu\text{m}$ -mesh screens (20  $\mu\text{m}$ ).

<sup>b</sup> SD, one standard deviation.

microflagellate, *Ochromonas* sp., selectively removed bacterial cells  $>0.2 \mu\text{m}^3$  in biovolume, and Turley et al. (24) found that two species of bacterivorous ciliates initially removed larger rod-shaped bacteria in preference to the smaller coccoid cells in the assemblage. Direct comparison of uptake of larger versus smaller fluorescently labeled bacteria (FLB) has also shown a positive relation between clearance rate and size of the prey cell. Gonzalez et al. (8) found that mixed-species assemblages of marine flagellates cleared 0.08- $\mu\text{m}^3$  FLB four times faster than they cleared 0.03- $\mu\text{m}^3$  FLB. Chrzanowski and Simek (6) reported that grazing rates by four species of freshwater flagellates were 2 to 18 times faster for 1.2- $\mu\text{m}^3$  FLB than for 0.4- $\mu\text{m}^3$  FLB.

In addition to the above empirical data, Monger and Landry (12) proposed a theoretical force-balance model for direct interception feeding by bacterivorous flagellates in which clearance rates ( $Cl_r$ ) for bacterial sized prey are positively related to the radius of the prey cell ( $R_p$ ), described by the general relationship  $Cl_r = aR_p^{0.8}$ . Their model predicts that a bacterivorous flagellate would graze 0.20- $\mu\text{m}^3$  (0.7- $\mu\text{m}$ -diameter) prey cells 1.8 times faster than it would graze 0.02- $\mu\text{m}^3$  (0.3- $\mu\text{m}$ -diameter) prey cells. In a subsequent study, Monger and Landry (13) concluded that comparison of actual clearance rates of large versus small microspheres or FLB by several species of flagellates supported the force-balance model.

In this study, high abundances of phagotrophic flagellates and small ciliates provided intense grazing pressure on the bacterioplankton assemblages in the water. The increases in total bacterial biovolume (number  $\times$  average cell size) in nongrazed treatments relative to those in grazed treatments were 3.3:1 in experiment I, 2.7:1 in experiment II, and 1.7:1 in experiment III (Table 2). The difference in grazing impact between experiments I and II and experiment III may have been related in part to differences in the incubation temperature (30°C for experiments I and II, 25°C for experiment III) and the growth rate of bacterivorous protists. In experiment II, flagellate numbers increased by a factor of 4.6 within 24 h, whereas, in experiment III, flagellate numbers only doubled overnight (Table 1). A higher biomass production for the protists would imply more intense grazing rates on the bacterial prey.

Bacterivory tended to decrease both the average bacterial cell size and FDC (Table 2, Fig. 1 through 3). The time courses of bacterial standing stock abundance and FDC showed an initial peak in FDC followed by an increase in

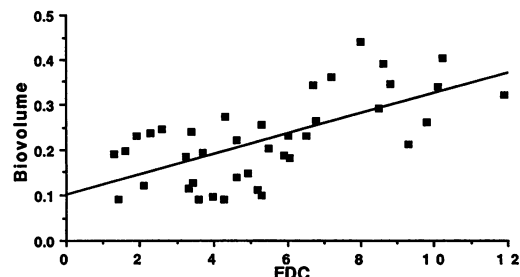


FIG. 4. Relation between average bacterial cell size and FDC for combined data from both size fractions and the three experiments. The equation for the linear regression is  $y = 0.104 + 0.021x$  ( $r = 0.65$ ).

bacterial biomass about 6 h later (Fig. 1 through 3). After the first 6 h of the experiments, total bacterial biomass, FDC, and average cell biovolume were all higher in nongrazed samples than in grazed samples (Table 2). FDC and bacterial cell size were positively related in these experiments (Fig. 4).

Our results do not imply that protist grazing decreases overall bacterial biomass production. A simple calculation of the estimated biovolume of bacteria consumed by flagellates and ciliates in experiments II and III may be made to compare bacterial production in the grazed and nongrazed treatments. The average cell sizes for the protist assemblages in these two experiments were about 5  $\mu\text{m}$  ( $50 \mu\text{m}^3$ ) for flagellates and 16  $\mu\text{m}$  ( $2,000 \mu\text{m}^3$ ) for ciliates. Assuming that the difference between the protist numbers at 0 and 24 h equalled the total production of protists and that the biovolume-based gross growth efficiency of the protists was 40%, we calculate that the protists must have consumed about  $1.36 \times 10^6 \mu\text{m}^3$  of bacterial biovolume per ml in experiment II and  $0.53 \times 10^6 \mu\text{m}^3$  of bacterial biovolume per ml in experiment III. Adding these estimated values to the measured bacterial biovolume increases (Table 2) yields revised values for bacterial production in grazed treatments of  $2.35 \times 10^6 \mu\text{m}^3/\text{ml}$  in experiment II and of  $1.22 \times 10^6 \mu\text{m}^3/\text{ml}$  in experiment III; these values are similar to the bacterial biovolume increases calculated for the nongrazed treatments (Table 2). Thus, the actual overall bacterial production in the grazed and nongrazed treatments must have been approximately equivalent.

It has been previously suggested that protist grazing may actually enhance the rate of bacterial cell growth. Sherr et al. (19) reported that grazing stimulated the rate of bacterial decomposition of polysaccharide detrital material. Sieburth and Davis (21) proposed that bacterivorous protists nurture the growth of their prey via producing labile organic compounds for bacterial uptake. Sherr et al. (18) found evidence for enhancement of bacterial growth in the presence of protist grazers as a result of increased ammonium recycling. The present study, however, was designed to investigate whether protists selectively crop bacterial biomass production, not whether protistan grazing changes the intrinsic growth rate, and thus the rate of production, of the bacterial assemblage.

Our initial hypothesis that protistan bacterivory can control in situ bacterial standing stock abundances by selectively cropping the larger, growing and dividing bacterial cells appears to be supported by the results of these experiments. The observed lag period between the initial increase of FDC and the subsequent increase in bacterial abundance

suggests that natural bacterioplankton assemblages must accumulate dividing cells before cell production results in higher bacterial standing stocks. This would be the pattern expected if bacterivores were keeping dividing cells at a low level in situ.

Other processes (for example, viral infection [5, 17]) might also contribute to low FDC and small bacterioplankton cell size by lysing proportionally more of the rapidly growing cells. Also, there may not always be a positive relationship between bacterial cell size and growth rate in situ. Nonetheless, the results reported here support the idea that selective protistan grazing is a major factor in explaining the low standing stock abundance, low FDC, and small average cell size typically reported for assemblages of suspended bacteria in both fresh and salt water (1, 8, 9, 11, 14).

More intense grazing pressure on larger, actively growing cells might also explain to some extent the coexistence of fast- and slow-growing bacteria in situ (3, 23). In the simplest form of the microbial loop concept, in which bacterivory is nonselective (3, 4), slower-growing cells should not be able to persist. Selective cropping of the more rapidly growing cells, which would normally exhibit a high FDC, would give a competitive advantage to the slow-growing degraders of recalcitrant organic matter (23). Thus, selective grazing of dividing cells may be an important factor in maintaining taxonomic and metabolic diversity within bacterioplankton assemblages.

#### ACKNOWLEDGMENTS

We thank Robin Krest for sizing the bacterial cells for experiment III.

This work was supported by National Science Foundation grants OCE-8816428 to E. Sherr and B. Sherr and OCE-8823091 to B. Sherr and E. Sherr as well as by grants from the Sapelo Island Research Foundation.

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