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Direct and Indirect Evidence of Size-Selective Grazing on Pelagic Bacteria by Freshwater Nanoflagellates

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Size-selective grazing of three heterotrophic nanoflagellates (with cell sizes of 21, 44, and 66 μm³) isolated from Lake Arlington, Texas was examined by using a natural mixture of fluorescence labelled lake bacteria. Sizes of ingested bacteria in food vacuoles were directly measured. Larger bacterial cells were ingested at a frequency much higher than that at which they occurred in the assemblage, indicating preferential flagellate grazing on the larger size classes within the lake bacterioplankton. Water samples were collected biweekly from June through September, 1989, fractionated by filtration, and incubated for 40 h at in situ temperatures. The average bacterial size was always larger in water which was passed through 1-μm-pore-size filters (1-μm-filtered water) (which was predator free) than in 5-μm-filtered water (which contained flagellates only) or in unfiltered water (in which all bacterivores were present). The increase of bacterial-cell size in 1-μm-filtered water was caused by a shift in the size structure of the bacterioplankton population. Larger cells became more abundant in the absence of flagellate grazing.

Protozoa, especially heterotrophic nanoflagellates, are recognized as major consumers of bacteria in both marine and fresh waters (3, 4, 10, 18, 22). Protozoa probably not only control bacterial abundance, but also affect the structure of the bacterial community via size-selective grazing. Field studies have shown that the average cell size of lake bacterioplankton decreases when under protozoan grazing pressure (14) but increases after removal of protozoans (27). Similar results were reported for a chemostat study, in which an *Ochromonas* sp. selectively grazed bacterial cells larger than 0.2 μm³ (2). Using fluorescence-labelled bacteria, Gonzalez et al. (9) found that a natural mixture of protozoa (both flagellates and ciliates) preferentially ingested the larger bacterial cells in a mixed population. In that study, the flagellates showed a pronounced response to bacterial size, with a threefold-higher grazing rate on large bacteria (0.08 to 0.10 μm³) than on small bacteria (0.03 μm³).

Although size-dependent grazing by flagellates has been strongly linked to the control of bacterial community size structure (1, 2, 7, 9), there is considerable uncertainty as to the nature and the degree of this relationship. Monger and Landry (19) recently reported that flagellate feeding varied as a function of prey size, that is, clearance rate varied roughly in direct proportion to the radius of the prey. The extent to which small size provides refuge from protistan grazing among the picoplankton might be less significant than was suggested by Gonzalez et al. (9), and therefore the role of the most minute bacteria in pelagic food webs is still questionable.

We previously reported (7) that flagellates, regardless of their size, preferred to feed on relatively large bacteria (*Pseudomonas* sp., between 0.8 and 1.2 μm³). However, the bacteria used as the prey in these single-species prey experiments were grown in chemostats and, for the most part, did not have the size distribution typical of natural bacterioplankton.

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The emphasis of the present study was to confirm that the size-selective flagellate-feeding pattern found for chemostat-grown bacteria was also true for natural bacterioplankton. Size-selective feeding was examined directly by measuring the cell sizes of bacterioplankton within food vacuoles of three contact-feeding flagellates. In field studies, the grazing impact was evaluated indirectly by monitoring changes in the mean cell volume and in the size class structure of the lake bacterioplankton population in the presence or absence of grazing.

MATERIALS AND METHODS

Sampling. Water samples were collected from Lake Arlington, Texas (32°42′32″N, 97°12′30″W; monomictic; surface area, 885 ha; maximum depth, 20 m; described by Chrzanowski [6]) with a 2- or 4-liter Van Dorn bottle. All samples were collected at depths of between 0.6 and 1.0 m, dispensed into 20-liter carboys, and transported to nearby laboratory facilities.

Size separation experiments. Water was fractionated into various size classes by using a filtration method similar to that described by Wright and Coffin (29). Preparations of duplicate bottles containing unfiltered and filtered water (5- and 1-μm-pore-size polycarbonate filters) were incubated for 40 h in the dark at in situ temperatures (water bath). Bacterial and protozoan abundances were determined at 10-h intervals (8), and at 0, 20, and 40 h, 40 bacterial cells were measured (see below) for each bottle (i.e., 80 cells per sample time).

Flagellate predators. Three colorless flagellates (designated I_3, I_5, and I_7; Table 1) were isolated from Lake Arlington (7). Two (I_3 and I_5) were bodonids, and one (I_7) was a chrysomonad. The flagellates were maintained on barley seed infusions (one barley seed autoclaved in 100 ml of filtered [pore size, 0.4 μm] lake water) inoculated with bacteria from flagellate-free lake water.

Size selection grazing experiments. Flagellates (from maintenance cultures) were gently concentrated from 50 to about

TABLE 1. Characteristics of flagellates and bacteria used in size selection studies

Flagellate	Size (μm^3) (mean ± SD) of:		Tracer (% of total)	Grazing rate (bacteria flagellate^{-1} h^{-1})
	Flagellates (n = 40)	Bacteria (FLB) ingested[a] (n = 100)		
I_3	61.0 ± 27.6	0.31 ± 0.18	22.0	20.2
I_5	44.6 ± 15.5	0.45 ± 0.30	19.4	29.7
I_7	21.0 ± 9.0	0.28 ± 0.16	23.0	6.7

[a] Bacteria offered to each type of flagellate had a mean size of 0.25 μm^3 (standard deviation, 0.22 μm^3)(n = 100).

5 ml by gravity filtration on filters (pore size, 2 μm). The flagellates were washed by gravity filtration three times with 15 ml of bacterium-free (i.e., passed through a 0.2-μm-pore-size filter [0.2-μm-filtered]) lake water. This procedure removed most of the bacteria originating from the maintenance cultures. Flagellates contained in the upper 2 ml of the remaining 5 ml of the last wash were inoculated into 200 ml of 1-μm-filtered lake water (in which only bacteria were present). These cultures were allowed to incubate undisturbed for 18 h. Simultaneously, bacteria from 2 liters of 1-μm-filtered lake water used to adapt flagellates to lake bacteria (above) were concentrated with a hollow-fiber filtration apparatus (Microgon, Minikros KF-200-003), fluorescently labelled (25) with 5-([4,6-dichlorotriazin 2-yl]amino) fluorescein (DTAF), and measured (100 cells).

Concentrations of fluorescence-labeled bacteria (FLB) were adjusted to between 19.4 and 23% of the total bacteria available as prey and fed to flagellates (Table 1). Subsamples (2 ml) were taken at 5-min intervals for 25 min and immediately fixed in an equal volume of 4% ice-cold glutaraldehyde (24). After 15 min of feeding, FLB contained in food vacuoles of randomly selected flagellates were counted and measured (100 FLB measured). Clearance rates, based on tracer consumption after 15 min of feeding, were calculated for various size categories of bacteria and used for calculation of a selectivity index (28; for details, see reference 7).

Staining, enumeration, and size measurements. Flagellates were stained and enumerated by using a modification of the primulin-staining procedure (5) as modified by Chrzanowski and Šimek (7). The modification produced excellent contrast between flagellates and FLB contained in food vacuoles.

Bacteria were enumerated from duplicate acridine orange-stained preparations of samples preserved with 2% formaldehyde (11).

All size measurements (of both flagellates and bacteria) were made with an eyepiece micrometer at a magnification of ×1,875. Volumes of organisms were calculated by the formulas reported in Chrzanowski and Šimek (7).

RESULTS

Direct evidence of size selective feeding by flagellates. Numbers of FLB in food vacuoles of flagellates increased linearly during the feeding period, with no obvious evidence of satiation (Fig. 1). Food vacuoles contained from one to eight FLB at the end of 15 min, and essentially no superimposition of ingested FLB in food vacuoles was observed. Grazing rates, determined from linear regression analysis (Table 1), were not proportional to the sizes of flagellates. The medium-sized flagellate (I_5) grazed the lake FLB at a higher rate than did the largest flagellate (I_3; Fig. 1).

Generally, the FLB ingested by flagellates were larger

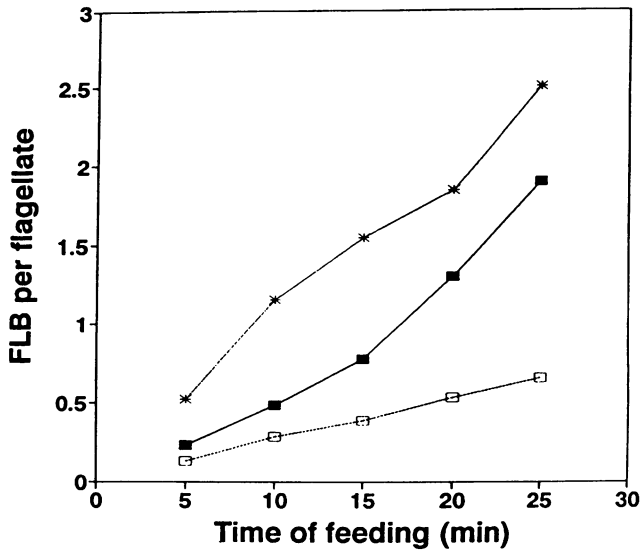

FIG. 1. Uptake of the DTAF-stained lake bacteria (FLB) by three heterotrophic nanoflagellates of various sizes (I_3 [■], I_5, [*], and I_7 [□]) (61 μm^3, 44 μm^3, and 21 μm^3, respectively).

than the average size of the FLB offered (Table 1 and Fig. 2). The size class distribution of the lake FLB offered was heavily skewed toward smaller cells (the most frequently occurring FLB were ≤0.1 μm^3 [Fig. 2]), yet the flagellates preferentially grazed cells in the larger size classes (Fig. 3). One-way analysis of variance on log-transformed data revealed highly significant differences (df = 3;396, F = 23.74, $P < 0.01$) among the average sizes of cells offered and ingested. Further analysis (multiple comparison of means, Student-Newman-Keuls test, $\alpha = 0.01$ [30]) revealed that the mean size of prey offered was significantly smaller than the mean size of prey ingested by any (all) of the flagellates (see below).

The pattern of the size selection did not differ significantly between flagellates I_3 and I_7 (Student-Newman-Keuls test, $\alpha = 0.01$). These flagellates took FLB between 0.2 and 0.4 μm^3 at higher frequencies than they took FLB of all other size classes. Bacteria falling between 0.3 and 0.5 μm^3 were ingested most frequently by I_5. I_5 was the strongest bacterivore (Table 1) and had the broadest range in the sizes of cells ingested. The distribution of cells ingested by I_5 was significantly different from the distribution of cells ingested by I_3 and I_7 ($\alpha = 0.01$). Selectivity indices (Fig. 3) indicated that I_5 had the highest preference for bacteria in the size categories 0.5 and 0.6 μm^3. The strongest preferences were calculated for bacteria in the 0.6-μm^3 category and for the largest bacteria (≥0.9 μm^3), where only 2% of the total tracer population was available (see below and Fig. 2).

The flagellates I_3 and I_7 were more selective than I_5. They also preferred to graze upon bacteria in size classes larger than the most frequent size class offered, but not on the largest size class available. These two flagellates, regardless of their very different sizes, preferred to feed on the same size classes of bacteria. They removed bacteria falling in the 0.3, 0.4, and 0.6-μm^3 categories with greater frequency than these cells appeared in the population. For example, cells in the 0.3-μm^3 size category composed 13% of available prey but constituted 34% (for I_3) and 37% (for I_7) of cells ingested. Selectivity indices clearly indicated a strong preference for

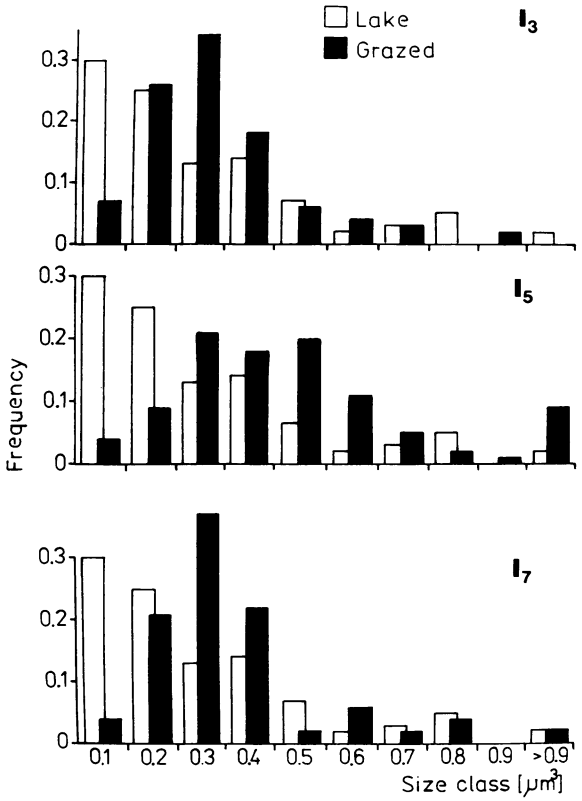

FIG. 2. Size class distribution of the lake bacteria (FLB) offered (open bars) and ingested (filled bars) by three heterotrophic nanoflagellates of various sizes (I_3, I_5, and I_7).

bacteria in 0.3- and 0.6-μm^3 categories (Fig. 3). The results for the 0.6-μm^3 category are based on a very small number of measurements, constituting only 2% of the total tracer available and 4% (I_3) and 6% (I_7) of the prey ingested. Because of the low frequency of the tracer, there is a large statistical uncertainty associated with these data. A similar situation existed with I_5's apparent preference for cells >0.9 μm^3 in size.

Indirect evidence of size-selective feeding by flagellates.

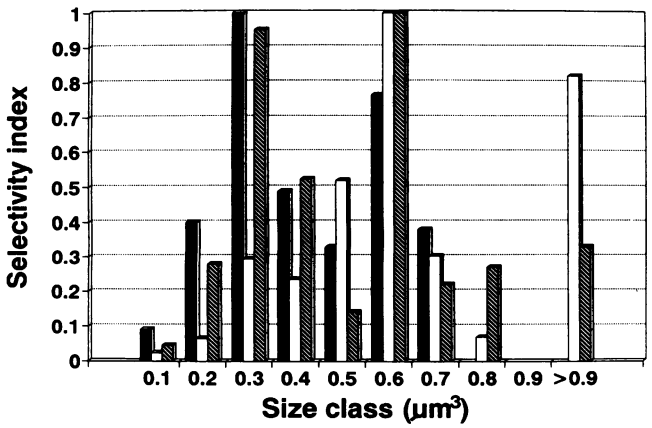

FIG. 3. Selectivity indices for each size class of prey for the heterotrophic nanoflagellates I_3 (■), I_5 (□), and I_7 (▨).

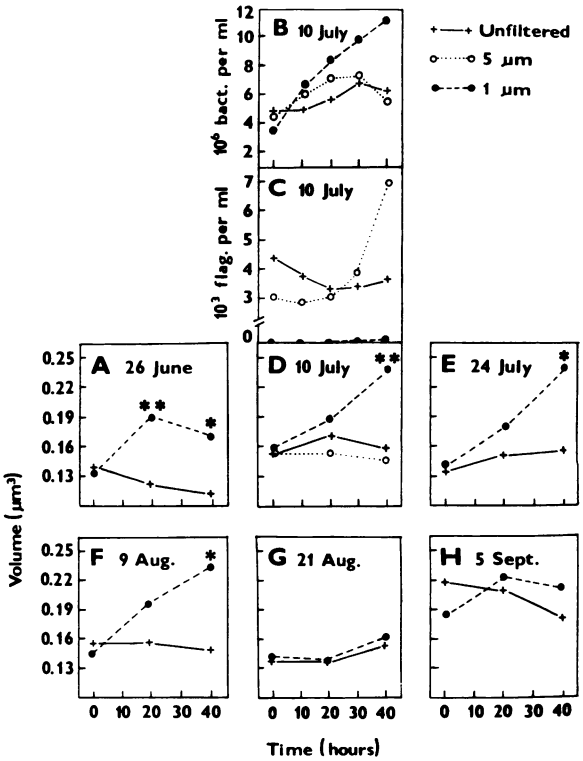

FIG. 4. Changes in mean bacterial volume through 40 h of incubation when all bacterivores were present (unfiltered lake water) and when all bacterivores were absent (1-μm-filtered lake water) for six sampling dates during the summer of 1989. Data from 10 July are supplemented by data for changes in mean bacterial volume in 5-μm-filtered lake water (containing only flagellates and bacteria) and for changes in bacterial and flagellate abundance determined at 10-h intervals (B and C). Asterisks indicate 1% (**) and 5% (*) significance levels (determined by *t* test) of differences in mean bacterial volumes in unfiltered and 1-μm-filtered samples.

Changes in the mean cell volume of bacteria during 40-h incubations were measured during experiments conducted every two weeks throughout the summer. Figure 4 illustrates a typical pattern of bacteria-flagellate grazing interactions in these experiments. In the absence of flagellates (1-μm-filtered samples), a roughly linear increase in bacterial abundance paralleled an increase in bacterial cell volume (Fig. 4; compare panels B and D).

The cell volume of bacteria in 1-μm-filtered samples always increased during the 40-h incubation period (Fig. 4A and D through H). The increase was gradual, and at the end of the incubation, very few flagellates were found to contaminate these preparations (<190 cells ml^{-1} at 40 h; data not shown except for 10 July [Fig. 4C and D]). This level of contamination was <7% of the flagellate abundance in unfiltered samples for the same time interval. If bacterial cell size decreased, it was concomitant with an increase in the abundance of contaminating flagellates (Fig. 4C and D). This pattern is apparent in the data from 26 June and 5 September (Fig. 4A and H [1-μm-filtered samples]), in which flagellates are shown to have grown to considerable levels by the end of the experiments (final flagellate abundance on 26 June was 3×10^2 ml^{-1}, and final flagellate abundance on 5 September was 1.89×10^3 ml^{-1}). A slight decrease in the mean cell volume occurred between 20 and 40 h parallel to an increase

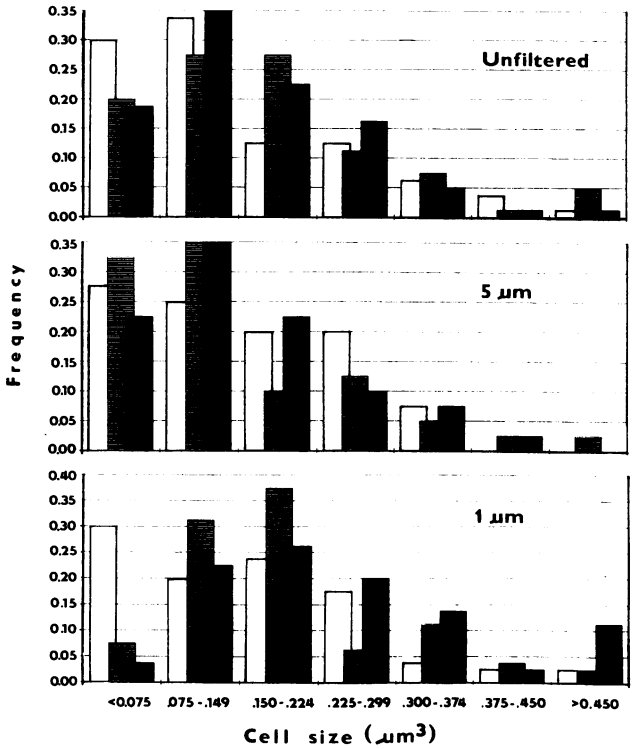

FIG. 5. Changes in size distributions of bacterial cell volumes in unfiltered, 5-µm-filtered, and 1-µm-filtered lake water after 0 (□), 20 (▤), and 40 (■) h of incubation (data from 10 July).

in the abundance of flagellates after 30 h. Changes in the mean cell volume of bacteria in unfiltered samples were slight throughout the incubations. However, the mean cell volume of bacteria in 1-µm-filtered incubations (Fig. 4) significantly increased in four of six size separation experiments. In one incubation (Fig. 4G), the increase was insignificant without apparent flagellate involvement, and in the other (Fig. 4H), the increase was insignificant because of heavy flagellate contamination.

In 5-µm-filtered lake water, the predator-prey system was simplified to encompass only flagellates and bacteria. The number of flagellates increased exponentially (Fig. 4C), and after 20 h of incubation, bacterial abundance (Fig. 4B) and average cell volume (Fig. 4D) began to decrease. In unfiltered water, flagellate abundance decreased while bacterial abundance increased for most of the incubation period. This result was likely brought about by the regulation of flagellate activity by larger flagellates, ciliates (rarely abundant in Lake Arlington), and perhaps metazoans remaining in the unfiltered water.

Our data also allow us to examine the changes in the size class distribution of the bacterial assemblage in unfiltered water (in which all bacterivores were present), in 5-µm-filtered water (in which flagellates were the only bacterivores), and in 1-µm-filtered preparations (which were predator free). Figure 5 depicts the size class distributions of bacteria at various time intervals for each of the preparations. Regardless of the incubation time, between 25 and 35% of the total bacteria in unfiltered and in 5-µm-filtered lake water fell into the modal size category from 0.075 to 0.145 μm^3. However, in predator-free preparations, there was a significant shift in the mean bacterial volume after 40 h (Fig. 4D; $P < 0.01$ [t test]) as well as in the size class structure (Fig. 5). The frequency of bacteria in the smallest size category (≤ 0.075 μm^3) fell from 30% at the beginning of the experiment to only about 4% at the end of the experiment. The modal size of cells shifted upward to the 0.150 to 0.224 μm^3 category. In addition, there was an increase in the frequency of bacteria in most size categories larger than the mode. The greatest increase in a single category occurred for cells in the ≥ 0.450-μm^3 category. Initially, only about 2% of the bacteria fell in this size class, but after 40 h, about 12% of the bacteria were in this category.

Statistical analyses revealed no significant differences between the data from unfiltered and 5-µm-filtered samples, while the mean cell size in 1-µm-filtered preparations was significantly higher than that in the others (determined by analysis of variance of log transforms [df = 2;197, $F = 15.21$, $P < 0.01$] followed by the Student-Newman-Keuls test [$\alpha = 0.01$]). These data alone suggest that flagellates are the principal bacterivores responsible for maintaining the relatively stable size structure of the bacterial assemblage in the lake water.

DISCUSSION

These data support the results of previous studies (2, 9) and clearly demonstrate that nanoflagellates preferentially ingest the larger bacterial cells in a mixed bacterioplankton assemblage. Our approach, while having the advantage of direct comparison of the size class distributions of bacterioplankton offered and ingested by the flagellates, has the potential disadvantage of working with heat-killed cells.

Landry et al. (16) and Monger and Landry (20) have demonstrated that live FLB were ingested at higher rates than were heat-killed FLB by a marine flagellate that feeds by direct interception. The heat-killing process may eliminate important chemical cues by which flagellates find their corresponding live prey and thereby artificially enhance the importance of FLB size as a selection parameter. Considerable evidence suggests that this source of bias is not likely to influence our conclusions.

When actively growing bacterioplankton populations are freed from predation pressure, the average cell size usually increases (e.g., see references 17 and 27) (Fig. 4 and 5). The increase in cell size is tightly coupled to an increase in bacterial abundance. While it may be argued that these increases result from enrichment of the water by nutrients released from organisms damaged during filtration and handling, the same trends were nevertheless found when bacteria were examined in undisturbed water samples. Krambeck (14) and Kuuppo-Leinikki (15) found that the average cell size of bacterioplankton decreased during periods of intensive grazing pressure.

Another, indirect line of evidence indicating a strong impact of flagellates on the size structure of bacterioplankton comes from our own data (Fig. 4). When flagellates were absent from 1-µm-filtered lake water, bacterial cell volume increased in essentially a linear manner during the course of the incubation. On two occasions (26 June and 5 September), flagellates heavily contaminated 1-µm-filtered samples, and their growth resulted in decreases in average bacterial cell volume by the end of the experiments. Additionally, only slight changes in bacterial size structure were observed in unfiltered and 5-µm-filtered lake water during the same period (Fig. 5). As the predator-prey system in 5-µm-filtered water was simplified to contain only flagellates and bacteria,

we can conclude that the greatest grazing impact on the size structure of the bacteria was due to flagellate grazing.

The idea that size selection of bacterial prey is a function of flagellate cell size (i.e., that the smallest phagotrophic flagellates tend to graze on the smallest bacterial cells) has been tested already. Gonzáles et al. (9) showed that there was virtually no shift in the size frequency distribution of a natural assemblage of bacterivorous flagellates responsible for grazing on bacterial prey whose cell volume varied by more than 1 order of magnitude. We also did not find any clear relationship between the size of flagellate predators and the size of the bacterial prey preferred. Of the three flagellates we studied, the medium-sized flagellate (I_5) appeared to have both the highest predation rate (Fig. 1 and Table 1) as well as the strongest preference for larger size classes of bacteria (Fig. 2).

Our direct measurements of size-selective grazing by isolated freshwater flagellates (Fig. 2 and 3) and indirect evidence from natural flagellate assemblages (Fig. 4 and 5) indicate that flagellates have a significant impact on the size structure of lake bacterioplankton. Thus, we conclude that natural assemblages of flagellates are size selective and responsible for the cell sizes typically found among lake bacteria. Similar results were predicted from a theoretical model (19) and confirmed by empirical data from both marine and freshwater systems (this study).

It seems reasonable to conclude that phagotrophic flagellates are responsible for maintaining a relatively stable bacterioplankton size structure (usually dominated by small bacterial cells [7, 9]). However, the extent to which small bacterial size provides a refuge from grazing must still be evaluated (e.g., compare references 7, 9, and 19).

That flagellate predators prefer large bacteria suggests an interesting scenario for changes in the taxonomic structures of bacterioplankton communities. Höfle (12) recently described a newly developed approach allowing for the determination of the taxonomic structure (to the species level) of natural microbial communities. Using direct analysis of RNA in bacterioplankton, he showed (13, 13a) that high genotypic diversity during the spring algal bloom was reduced significantly by intensive grazing by heterotrophic nanoflagellates. The strong shift in bacterioplankton species composition might be explained by a combination of substrate availability and size-selective grazing. If the larger cells within natural bacterial populations represent the rapidly growing segment of the population (acting similarly to those in culture [21, 23]), then increased grazing pressure upon large, actively growing and dividing bacterial cells should lead to very strong selection among bacterial strains. Only those strains capable of balancing grazing losses and growth rate will maintain a presence in the population. On the other hand, with decreasing grazing pressure and the same substrate availability, there is a higher probability that even cells of more slowly growing bacterial strains could enlarge enough to reach the predivision stage. Thus, preferential flagellate grazing upon large bacteria not only might crop bacterial production (9) but also might control the species diversity of the bacterioplankton community. Recent data appear to support at least part of this hypothesis. Sherr et al. (26) recently demonstrated that protistan bacterivory can control in situ bacterial standing-stock abundance by selective cropping of the larger growing and dividing cells.

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