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Differential feeding by marine flagellates on growing versus starving, and on motile versus nonmotile, bacterial prey

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ABSTRACT: Previous work has demonstrated selective feeding by bacterivorous protists on the basis of prey size. Other prey characteristics may also affect protist grazing rates. In this study, we investigated relative uptake rates of fluorescently labeled bacterial (FLB) cells made from growing (G-FLB) vs starving bacteria (S-FLB), and of live-stained motile vs non-motile bacteria by marine phagotrophic flagellates. We also determined relative growth rates and digestion times for flagellates fed growing or starved bacteria. A natural assemblage of marine flagellates and a flagellate isolate, *Cafeteria* sp., showed lower ($p < 0.001$) clearance rates on S-FLB than on G-FLB, which could be ascribed to size-selective grazing by the flagellates. Moreover, flagellates showed longer ($p < 0.001$) prey digestion times when feeding on S-FLB than on G-FLB. The carbon-based gross growth efficiency of flagellates fed starved bacteria was nearly twice that of flagellates fed growing bacteria: 38.5% and 21.5%, respectively. Uptake rates by flagellates of several live-stained bacteria (LSB) of either motile or non-motile strains were compared with uptake rates of heat-killed FLB made from the same bacterial cultures. Clearance rates were significantly higher for LSB compared to FLB only for motile strains; no differences in uptake rates were observed for non-motile bacteria. Although FLB may be a good tracer for non-motile bacteria, use of FLB may lead to underestimation of actual grazing rates if motile bacteria comprise a significant fraction of the total bacterial assemblage. Our results suggest (1) grazing on starved bacteria results in a more efficient transfer of biomass to higher trophic levels than does grazing on growing bacteria; (2) use of motile LSB in prey uptake experiments should yield higher estimates of bacterivory compared to rates estimated using heat-killed or non-motile labeled prey; and (3) qualitative factors affecting predator-prey dynamics may be important in regulating elemental fluxes in microbial food webs.

KEY WORDS: Bacterivory · Flagellate · Microbial loop

INTRODUCTION

The impact of protist grazing on bacterial standing stocks in aquatic ecosystems has been widely recognized (e.g. Azam et al. 1983, McManus & Fuhrman 1988, Pace 1988). If protistan grazing is routinely the major source of bacterial mortality, then there should be, on average, a balance between production of bacterial cells and loss of bacterial cells due to predation (Pace 1988, Sherr et al. 1989, Bloem et al. 1988). Heterotrophic protists, primarily flagellates in the nanoplankton size range (2 to 20 µm), have been identified as major grazers of bacteria in pelagic ecosystems (Fenchel 1982b, Azam et al. 1983, Sherr & Sherr 1984).

Microorganisms live in a changing environment (Fenchel 1982b, Azam & Ammerman 1984, Morita 1985, Nyström et al. 1990). If substrates are available, bacteria grow. When substrates are depleted, bacteria show an adaptive response termed starvation-survival (Morita 1985, Morita & Moyer 1989, Nyström et al. 1990). Starved bacteria have different physical and biochemical properties compared to growing bacteria. Starvation causes a several-fold decrease in biovolume

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of bacterial cells (Marden et al. 1985, Morita 1985, Moyer & Morita 1989, Nyström et al. 1990). In addition, growing and starved bacteria differ in composition of lipids and cell envelopes (Chai 1983, Guckert et al. 1986, Hood et al. 1986, Gauthier et al. 1989), in protein composition and in RNA and DNA content (Hood et al. 1986, Jaan et al. 1986, Morita & Moyer 1989, Nyström et al. 1990). Simon & Azam (1989) reported that marine bacterioplankton <0.10 μm^3 in size had higher protein : biovolume and carbon : bioviolume ratios than did larger cells growing in nutrient-amended bacterial assemblages.

Moreover, individual species of bacteria can be either motile or non-motile. Motile bacteria have the advantage of being able to move to substrate-rich microzones, while non-motile bacteria have to depend on ambient nutrient conditions and/or external sources of nutrients (Azam & Ammerman 1984, Mitchell et al. 1985, Jackson 1987, Azam & Smith 1991, Lauffenburger 1991). Several authors (Macnab & Koshland 1972, Koshland 1974, Jackson 1987, Lauffenburger 1991) have reported chemosensory mechanisms in motile bacteria. If motile bacteria can move to areas with higher substrate concentrations compared to ambient, they should be able to grow faster than non-motile bacteria. In support of this idea, Lauffenburger (1991) showed that motile bacteria can outcompete non-motile bacteria in a heterogeneous, oligotrophic marine system.

On the basis of the above considerations, it would be logical to suppose that rates of protist predation might be affected by the relative growth state and motility of the bacterioplankton assemblage. Such differential grazing might be important as a feedback mechanism within microbial food webs. For instance, a lower grazing pressure on starved/non-motile bacteria vs growing/motile bacteria would lead to higher survival rates of slower growing compared to faster growing cells, thus permitting slow growing cells to persist in the bacterial assemblage. Results of previous studies suggest the potential for control of protistan grazing rates by qualitative characteristics of the bacterial prey. For example, Sherr et al. (1983) and Mitchell et al. (1988) reported differential growth yields among aquatic flagellates grown on several monospecific isolates of bacterial prey. González et al. (1990a) observed differential digestion times for individual species of enteric bacteria ingested by marine protists. Monger & Landry (1992) found higher flagellate clearance rates for a motile bacterium compared to clearance rates of non-motile bacteria.

This study was designed to investigate whether grazing rates of marine bacterivorous flagellates can be regulated by differences in growth state and motility of bacterial species. We hypothesized that there should be significant differences between flagellate grazing rates on growing (G bacteria) compared to starving bacteria (S bacteria), and also between grazing rates on motile vs non-motile bacteria. We analyzed whether flagellates showed variable grazing on G vs S bacteria prepared from the same bacterial isolate, and whether flagellates preferentially grazed motile compared to non-motile bacteria. We also compared the ability of flagellates to process G and S bacteria by conducting ingestion, digestion, and growth experiments comparing both types of bacteria. Grazing rates on motile bacteria and non-motile bacteria were determined by comparing flagellate uptake rates of live-stained bacteria (LSB) and heat-killed fluorescently labeled bacteria (FLB) made from several marine bacterial isolates. Our results suggested that phagotrophic flagellates do have differential feeding responses between growing and starving bacteria, and between motile and non-motile bacteria.

MATERIALS AND METHODS

Isolates. Several strains of marine bacteria were isolated on seawater culture (SWC) agar, and maintained on SWC agar or broth. The composition of SWC per liter of 75% artificial seawater was: 5 g Bacto-Peptone, 1 g Bacto-Yeast extract and 3 ml glycerol (Nealson 1978). A fast-moving bacterium (MB2A), a slow-moving bacterium (Y) and a non-motile bacterium (H1) were used in this study. A 3 to 5 µm bacterivorous flagellate, identified as *Cafeteria* sp., was isolated by the dilution method (Fenchel 1982a) and maintained at 15°C on 0.2 µm filtered seawater with 1 boiled wheat grain and a small inoculum of 0.8 µm filtered seawater per 100 ml. Both bacterial and flagellate isolates were obtained from surface water samples collected 5 km due west of Yaquina Bay (Oregon, USA). Seawater samples with natural flagellate assemblages were collected from Yaquina Bay or Coos Bay, Oregon.

Preparation of fluorescently labeled bacteria (FLB). Heat-killed FLB were prepared as described by Sherr et al. (1987). The Y bacterial isolate was grown on SWC broth at 25°C. Exponential phase bacteria (G bacteria) were harvested by centrifugation, $10\,000 \times g$ for 20 min at 4°C. Bacteria were suspended in either phosphate buffered saline solution (PBS) (Sherr et al. 1987) or nine salt solution (NSS) (Marden et al. 1985). PBS suspended cells were used to prepare G-FLB immediately upon harvesting. NSS suspended cells were washed twice, inoculated in NSS, and starved for 7 d at room temperature. After this period, starved bacteria (S bacteria) were harvested as above, and S-FLB were prepared. Bacterial assemblages were stained with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF)

at 60°C for 2 h, as described by Sherr et al. (1987). Unstained, heat-killed cells were also prepared from G and S bacteria as above, but without addition of the fluorochrome.

Preparation of live-stained bacteria (LSB). The live-staining protocol briefly described here is a modification of that of Monger & Landry (1992). Three bacterial isolates, MB2A, Y, and H1, were grown in SWC broth at 25°C without shaking. Cells were harvested as above in the exponential growth phase, washed twice with 0.2 µm filtered seawater and suspended in 0.2 µm filtered seawater for 2 d. For one-half of the suspension, bacteria were pelleted by centrifugation and suspended in the live staining solution: 100 µg ml^{-1} fluorescein isothiocyanate (FITC) and 40 µg ml^{-1} dithioerythritol dissolved in 0.2 µm filtered seawater. Both halves of the suspension were incubated overnight. Subsequently, live-stained bacteria were washed 3 times and suspended in 0.2 µm filtered seawater. The second half of the bacterial suspension was then harvested and processed as outlined above to make heat-killed FLB. Both FLB and LSB were used immediately after this preparation procedure was finished.

Enumeration procedures. Bacterial abundance was estimated by the acridine orange direct count method (Hobbie et al. 1977). Flagellates were enumerated by DAPI staining according to Porter & Feig (1980) as modified by Sherr et al. (1992). FLB and LSB were counted after settling of known aliquots of the labeled bacterial suspensions on unstained 0.2 µm polycarbonate filters. FLB and LSB in flagellate food vacuoles were visualized in DAPI stained preparations as described by Sherr et al. (1987). Bacterial volumes were estimated from enlarged micrographs projected onto a screen, using a projected image of a stage micrometer at the same magnification as calibration (Suzuki et al. 1993). Flagellate biovolumes were estimated by measuring the diameters of 50 to 100 individual cells in several samples taken during growth experiments. The C and N content of G and S bacteria was determined with suspensions of cultured cells, concentrated and washed via centrifugation. Small aliquots of the suspensions were used to determine bacterial cell abundance via epifluorescence microscopy. The rest of the suspensions were spotted onto precombusted GFF filters, which were then dried and analyzed for carbon and nitrogen content using a Perkin Elmer 240c CHN analyzer. Total flagellate carbon biomass was estimated using the carbon conversion factor 0.22 pg C μm^{-3}, which compensates for cell shrinkage upon preservation (Børsheim & Bratbak 1987).

Experimental protocols comparing feeding on G and S bacteria. All experiments were carried out in 400 ml Whirl Pak bags presoaked in 10% (vol/vol) HCl and copiously rinsed with deionized water (Sherr et al. 1987). Experiments were run at 15°C in the dark.

Ingestion and digestion experiments: A mixed culture of bacterivorous flagellates was obtained by incubating natural seawater with 1 boiled wheat grain per 100 ml for 4 d. 100 ml portions of the flagellate culture were transferred to 6 experimental Whirl Pak bags (100 ml per bag). Three bags were inoculated with G-FLB, and the other three with S-FLB. The final concentration of FLB was about 10^6 FLB ml^{-1}, approximately 10% of the abundance of live bacteria. Samples were taken at several times during a 2 h period after the FLB addition, fixed by the Lugol-Formalin decoloration technique (Sherr et al. 1992), and subsequently inspected for presence of FLB in flagellate food vacuoles.

FLB appearance/disappearance experiments were carried out following the protocol of Sherr et al. (1988). Briefly, after the number of FLB per flagellate cell reached a plateau, a 10-fold dilution of the experimental samples with 0.6 µm filtered seawater containing only non-stained bacteria resulted in a linear decrease in the average number of FLB per flagellate cell over time. Time 0 of the disappearance slope was taken as the time of dilution. Dilution controls, i.e. separate experiments in which only 10% of the initial amount of FLB used in the appearance/disappearance experiments was added, were carried out for each experiment to correct the FLB disappearance slopes (Sherr et al. 1988).

Rates of both appearance and disappearance of FLB in food vacuoles (increase or decrease in the average number of FLB $cell^{-1}$ min^{-1}) were determined via linear regression analysis as the slopes of the linear portions of the appearance and disappearance curves, respectively. Per-cell clearance rates (nl $cell^{-1}$ h^{-1}) were calculated from the uptake rates of FLB and the absolute concentration of FLB in each experimental bag (Sherr et al. 1987). Digestion times (minutes) were calculated from the x-intercept of the disappearance regression lines.

Growth on G and S bacteria: For these experiments, we used *Cafeteria* sp. Flagellates (final density of about 10^2 cells ml^{-1}) were added to 0.2 µm filtered seawater containing vancomycin (200 mg l^{-1}) and penicillin (1 mg l^{-1}) to inhibit growth of live bacteria (Sherr et al. 1986). The experimental bags were allowed to sit for 2 h in the incubator, and then either G- or S-FLB were separately added to duplicate bags to a final concentration of 10^8 FLB ml^{-1}. Periodically, subsamples from each bag were collected and fixed as above. Numbers and biovolumes of flagellates, and abundance of remaining FLB were determined for each sampling time. Growth rates were calculated from the equation: $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_1 and N_2 = average values of flagellate abundance at the begin-

ning and at the end, respectively, of exponential growth; and t_1 and t_2 = the corresponding times for N_1 and N_2. Doubling times were calculated as $(\ln 2)/\mu$ (Anderson 1988).

FLB appearance/disappearance experiments with flagellates fed growing or starved bacteria: These experiments were carried out in order to test whether the past feeding history of flagellates had any effect on clearance rates and/or digestion times of G-FLB vs S-FLB. *Cafeteria* sp. was grown as described above, except that unstained, heat-killed G or S bacteria were added as food. At mid-exponential growth phase, 100 ml aliquots of flagellate culture (about 10^4 cells ml^{-1}) were transferred to Whirl Pak bags. Nine experimental bags were prepared from the culture growing on G bacteria. Triplicate bags were inoculated with either G-FLB, S-FLB, or 0.5 µm diameter fluorescent microspheres (FM) (Polysciences, Inc.). The same experimental treatments were set up for the culture growing on S bacteria. Final concentrations of added fluorescent particles were from 1 to 7 × 10^6 ml^{-1}; on average, FLB was about 6% of the total concentration of bacteria, 5 to 9 × 10^7 ml^{-1}. Clearance rates and digestion times for G-FLB, S-FLB, and FM were determined following the protocols outlined above.

Experimental protocol comparing motile and non-motile bacteria. Three marine bacterial isolates – a fast-moving bacterium (MB2A), a slow-moving bacterium (Y), and a non-motile bacterium (H1) – were used in these experiments. LSB and FLB were prepared from each isolate, and the average bacterial cell biovolume of each preparation was determined. LSB and FLB made from each of the 3 isolates were separately added to duplicate samples of *Cafeteria* sp. in Whirl Pak bags. Ingestion rate assays for LSB and FLB made from each bacterial isolate were carried out as described above.

Estimating bacterial motility. Three different measures of motility were made for the 3 bacterial isolates: (1) The percentage of moving bacterial cells was estimated by counting in a hemocytometer the number of bacteria that were, at that moment, moving, i.e. running, but not tumbling, using the terminology of Koshland (1974), with respect to the total number of bacteria. (2) Unpreserved samples of bacterial culture were concentrated 1:100 by filtration through 0.2 µm polycarbonate filters. The speed (µm s^{-1}) of bacterial cells was determined from the time it took for individual motile cells to cross a 100 µm wide grid. (3) Bacterial motility was investigated on a different time scale by inoculating LSB in the center of a Petri dish with SWC semi-solid agar (0.6% agar) and incubating the plates at 25°C for 2 d. The radius (in mm) of the area showing visible growth was used as a relative estimate of bacterial motility.

Statistical analysis. Statistical analyses were performed as described by Sokal & Rohlf (1981). ANOVA and planned comparisons were used for comparing average values. *F*-tests were used to compare regression coefficients. In appearance/ disappearance experiments, Student's *t*-test was used to test whether slopes of FLB $cell^{-1}$ and FM $cell^{-1}$ were significantly different from zero.

RESULTS

FLB appearance/disappearance experiments using G and S bacteria

In the first series of experiments, we determined clearance rates and digestion times for natural assemblages of flagellates fed either G-FLB or S-FLB. For the natural assemblages, clearance rates were 3.4 to 3.7 times greater for G bacteria than for S bacteria (Table 1). The average cell biovolume of G bacteria (0.47 ± 0.25 μm^3) was 3.7 times larger than, and significantly different from ($p < 0.01$), the average cell biovolume of S bacteria (0.13 ± 0.09 μm^3). Both flagellate clearance rates and bacterial biovolumes were significantly different ($p < 0.001$) for the 2 types of FLB. Digestion times for G-FLB by natural assemblages of

Table 1. Clearance rates and digestion times of growing (G-) and starving (S-) fluorescently labeled bacteria (FLB), and digestion times of fluorescent microspheres (FM), by a mixed-species enrichment culture of marine flagellates, and by a marine flagellate isolate, *Cafeteria* sp., grown on growing (G) or starved (S) bacteria. Mean values ± 1 SD. Significance levels of differences in clearance rates between G- and S-FLB: *$p < 0.001$; ns: not significant

Flagellate assemblage	Clearance rates (nl $cell^{-1}$ h^{-1})			Digestion times (min)	
	G-FLB	S-FLB	FM	G-FLB	S-FLB
Enrichment culture	0.37 (0.05)	0.10 (0.02)*	–	66 (4)	156 (10)*
Enrichment culture	0.19 (0.02)	0.06 (0.01)*	–	55 (2)	104 (4)*
G-grown flagellate isolate	0.30 (0.01)	0.26 (0.01)*	0.25 (0.04)	63 (0)	295 (64)*
S-grown flagellate isolate	0.31 (0.00)	0.26 (0.01)*	0.22 (0.02)	70 (1)	93 (8)^{ns}

flagellates were from 1.9 to 2.4 times shorter than those for S-FLB (Table 1). The differences between digestion times were also significant ($p < 0.001$).

Flagellate growth experiments on G and S bacteria

In both treatments, added G-FLB or S-FLB formed the sole food resource; growth of live bacteria was inhibited by antibiotics, and initial abundance of live bacterial cells was 0.1% of the abundance of added FLB. The growth rate (μ) of *Cafeteria* sp. fed G-FLB was 0.18 ± 0.01 h^{-1} (doubling time of 3.9 h). *Cafeteria* sp. fed S-FLB had a μ of 0.13 ± 0.004 h^{-1} (doubling time of 5.2 h) (Fig. 1A). Flagellate cells growing on S bacteria were larger than those growing on G bacteria (Fig. 1B). Differences in cell biovolume were apparent beginning with $t = 48$ h ($p < 0.05$), just as the exponential phase of flagellate growth began, and were maintained during the rest of the growth curve (Fig. 1B). A larger proportion of G than S bacteria was consumed (Fig. 1C). By the end of the experiment, the final concentration of G bacteria was 8.6% of the initial amount, while 41.2% of initial numbers of S bacteria remained at the end. In order to compare the potential nutritional value of G and S bacteria, we calculated the C and N content of both types of bacteria. C and N per unit biovolume was 0.092 pg C μm^{-3} and 0.023 pg N μm^{-3} for G bacteria, and 0.208 pg C μm^{-3} and 0.054 pg N μm^{-3} for S bacteria. Thus, S bacteria had a C and N content per unit biovolume about 2.3 times greater, a significant difference ($p < 0.05$), compared to G bacteria. On a per-cell basis, the average elemental C and N content of the S bacteria was 27 fg C and 7 fg N, similar to values reported for marine bacterioplankton growing in seawater culture (Lee & Fuhrman 1987, Simon & Azam 1989); the corresponding values for the G bacteria were 44 fg C $cell^{-1}$ and 11 fg N $cell^{-1}$.

Combining these results with estimates of flagellate carbon yields, we calculated carbon-based gross growth efficiencies of 21.5 and 38.5% for flagellates growing on G and S bacteria respectively. The growth efficiencies were based on the difference between the amounts of carbon present in added prey cell biomass at the beginning and end of each experiment.

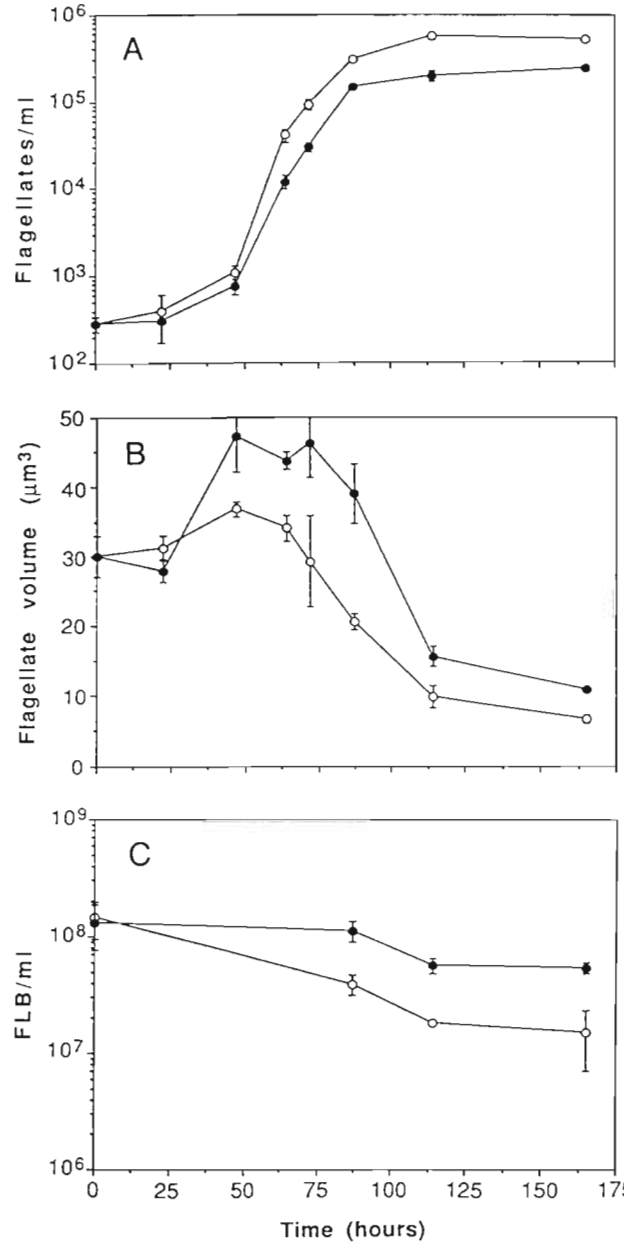

Fig. 1. (A) Flagellate abundance, (B) average flagellate biovolume, and (C) abundance of fluorescently labeled bacteria (FLB) in experiments performed with flagellates feeding on growing (o) and starved (•) FLB. Error bars indicate 1 standard deviation

Appearance/disappearance experiments with flagellates grown on G and S bacteria

In both treatments, i.e. *Cafeteria* sp. grown on either G bacteria or S bacteria, clearance rates were greater ($p < 0.01$) for G- than for S-FLB (Table 1). For the same FLB type, similar clearance rates were observed for flagellates grown on G or S bacteria (Table 1). No significant differences in clearance rates of FM were observed for flagellates fed G or S bacteria (Table 1).

Results of the FLB appearance/disappearance experiments with *Cafeteria* sp. are shown in Fig. 2. Digestion times were determined from the intercepts of the *x*-axis. Flagellates grown on G bacteria showed diges-

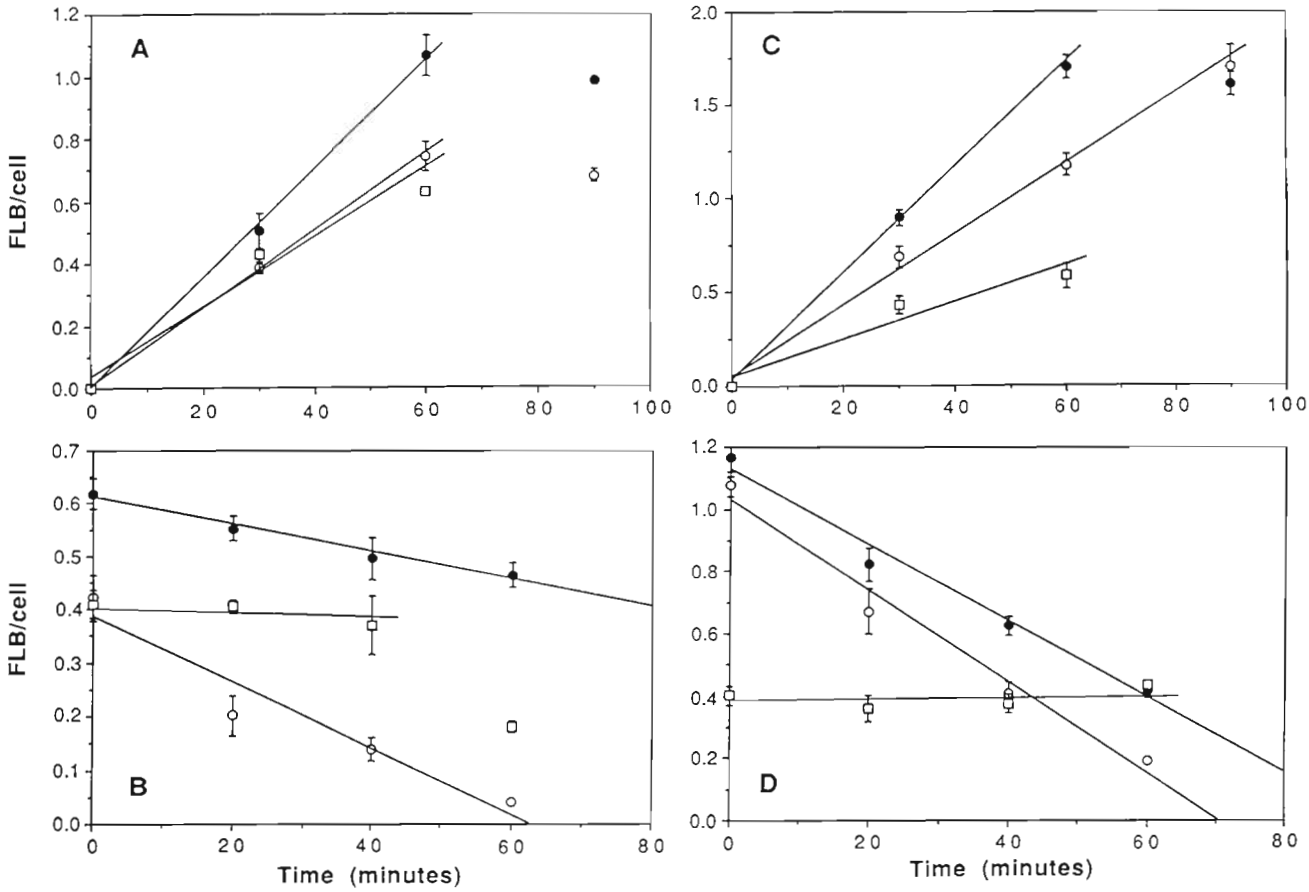

Fig. 2. Changes in number per flagellate cell over time of ingested growing FLB (○), starved FLB (●), and fluorescent microspheres (FM, □) in *Cafeteria* sp. feeding on (A, B) growing or (C, D) starved bacteria. Error bars indicate 1 standard deviation. (A) and (C) show increase in ingested prey cell^{-1} during the uptake phase of the experiments; (B) and (D) show decrease in ingested prey cell^{-1} after a 1:10 dilution of the cultures carried out after the uptake curves shown in (A) and (C) had leveled off. An observed rapid decrease in number cell^{-1} of FM after 60 min post-dilution for cultures fed starved bacteria is not shown

tion times significantly greater ($p < 0.001$) for S-FLB than G-FLB (Table 1). However, digestion times of flagellates grown on S bacteria were not significantly different for G- or S-FLB (Table 1). For G-FLB, digestion times were comparable for flagellates grown on both types of bacteria.

The slopes of FM cell^{-1} over time after dilution (0 to 40 min in Fig. 2B, and 0 to 60 min in Fig. 2D) were not significantly different from zero until a certain period of time had passed, after which FM rapidly disappeared from the flagellates. This observation indicated that FM were not randomly egested by flagellates, which agrees with the results obtained by Dubowsky (1974), and supports the idea of orderly processing of food vacuoles in bacterivorous flagellates. In our experiments, FM were retained for a shorter period of time by flagellates grown on G bacteria than by flagellates grown on S bacteria (Fig. 2B, D).

Ingestion experiments on motile and non-motile bacteria

The 3 strains of bacteria used in these experiments had markedly different degrees of motility. For the MB2A strain, a fast-moving bacterium, we estimated that from 15 to 23.5% of LSB were moving and that the average speed of the moving bacteria was 83 (SD = 22) μm s^{-1}. For the Y strain, a slow-moving bacterium, we observed only 1.5 to 2.5% moving LSB, and an average speed of 16 (SD = 5) μm s^{-1}. The H1 strain is a non-motile bacterium. We also observed that shaking the bacterial cultures at about 100 rpm on a rotatory table resulted in a loss of bacterial motility. For the MB2A strain, shaking the culture reduced the bacterial speed to 27 (SD = 4) μm s^{-1}, and the percentage of moving MB2A to less than 2%.

Measurements of bacterial motility on semi-solid agar plates for the 3 strains used in this study sup-

ported motility measures made by direct inspection of the bacteria. Bacterial growth was observed up to 14.5, 1.5 and <0.5 mm from the inoculation point for MB2A, Y and H1 LSB respectively.

No significant differences between biovolumes of LSB and FLB were found for either motile (MB2A and Y) or non-motile (H1) bacteria.

Clearance rates of LSB and FLB by *Cafeteria* sp. and by a natural assemblage of phagotrophic flagellates are shown in Table 2. FLB made from each of the strains were heat killed, thus non-motile, while live staining did not inhibit cell motility. No differences in clearance rate between LSB and FLB were found for the non-motile bacteria (H1 strain). However, significant differences ($p < 0.001$) were observed between LSB and FLB clearance rates when motile bacteria (MB2A and Y strains) were used. For the MB2A strain, clearance rates on LSB were 3.8- and 2.7-fold higher than clearance rates on FLB, for the natural assemblage and *Cafeteria* sp. respectively. For the Y strain, clearance rates on LSB were 2.2- and 2.6-fold higher than the respective clearance rates on FLB. For the non-motile H1 strain, the ratios of clearance rates of LSB/FLB were 1.03 and 1.04, i.e. not different.

Table 2. Clearance rates (± SD) by bacterivorous flagellates of live-stained bacteria (LSB) and of heat-killed, non-motile fluorescently labeled bacteria (FLB) made from 3 isolated marine bacteria: MB2A, a fast-moving strain; Y, a slow-moving strain; and H1, a non-motile strain. Clearance rates of LSB and of FLB were determined both for a natural flagellate assemblage in seawater collected on the Oregon coast and for a monospecific culture of a marine bacterivorous flagellate, *Cafeteria* sp. Significance levels of differences between clearance rates of LSB and FLB: $^{*}p < 0.001$; ns: not significant. n = 6

Bacterial isolate	Flagellate assemblage	Clearance rates (nl cell^{-1} h^{-1}) LSB	FLB	LSB:FLB
MB2A	Monospecific culture	2.32 (0.36)	0.61 (0.28)*	3.8
	Natural assemblage	1.71 (0.24)	0.63 (0.07)*	2.7
Y	Monospecific culture	0.82 (0.30)	0.31 (0.11)*	2.6
	Natural assemblage	1.68 (0.18)	0.78 (0.12)*	2.2
H1	Monospecific culture	0.41 (0.03)	0.40 (0.07)^{ns}	1.0
	Natural assemblage	0.54 (0.08)	0.52 (0.10)^{ns}	1.0

DISCUSSION

We investigated the impact of 2 qualitative aspects of bacterial prey, growth state and motility, on flagellate bacterivory. Growing and starved bacteria differ not only in biovolume (Marden et al. 1985, Morita 1985, Moyer & Morita 1989, Nyström et al. 1990), but also in biochemical characteristics (Kjelleberg & Hermansson 1984, Marden et al. 1985, Morita 1985, Guckert et al. 1986, Hood et al. 1986, Jaan et al. 1986, Morita & Moyer 1989, Nyström et al. 1990). In previous studies, positive relationships between bacterial cell size and protist clearance rates have been observed (Andersson et al. 1986, Chrzanowski & Simek 1990, González et al. 1990b, Monger & Landry 1990). In addition, González et al. (1990a) reported differential digestion rates for various bacterial species by phagotrophic flagellates. Less is known about effect of prey motility on bacterivory. Mitchell et al. (1988) found that a freshwater flagellate had lower growth yields when fed a motile bacterium compared to yields with non-motile bacteria as a food source. However, Monger & Landry (1992) reported higher clearance rates for a live-stained motile bacterium than for heat-killed FLB made from the same culture. In our study, we analyzed predation (1) on a single bacterial species under distinct growth conditions, and (2) on motile vs non-motile bacteria, both for a cultured marine flagellate and for natural assemblages of phagotrophic flagellates in seawater.

Grazing on G and S bacteria

The results of the clearance (Table 1) and growth (Fig. 1) experiments indicated that flagellates cleared G bacteria at a faster rate, and were able to consume a larger proportion of available G bacteria, than was found for S bacteria. This finding can be related to the fact that G bacteria (0.47 μm^3) are larger than S bacteria (0.13 μm^3). The size of the S bacteria used in this study was somewhat larger than the average cell size of most marine bacterioplankton, 0.05 to 0.10 μm^3 (e.g. Furhman 1981, Ammerman et al. 1984), but within the size range of bacterioplankton present in Oregon coastal waters (Suzuki et al. 1993). Other authors (Krambeck 1988, Chrzanowsky & Simek 1990, González et al. 1990b) have reported higher grazing rates by protists on larger compared to smaller bacterial cells. In addition, Sherr et al. (1992) found a greater grazing impact by protists on dividing cells compared to non-dividing cells in bacterioplankton assemblages.

Prey size is not the sole consideration in explaining faster clearance of G-FLB than S-FLB, however. Rate of predation is also dependent on how long a time is necessary for ingested cells to be digested. We found that S bacteria were digested more slowly (longer vacuole processing times) than were G bacteria by both *Cafeteria* sp. and by mixed species flagellate assemblages

(Table 1). Also, ingested fluorescent microspheres, which, in theory, are retained in the cell until vacuole contents are egested, were retained for a shorter period of time in flagellates fed G bacteria than in those fed S bacteria. This suggests a longer vacuole processing time is involved during the digestion of smaller, starved bacteria compared to the digestion of larger, growing bacteria. It is interesting that flagellates grown on S bacteria had a larger cell biovolume than those grown on G bacteria (Fig. 1B); this might imply that flagellates can maximize the throughput of difficult-to-digest prey biomass by increasing either the number or average size of food vacuoles in the cell.

We also found evidence for physiological adaptation of bacterivorous flagellates to starved bacterial prey. Both natural flagellate assemblages feeding on the 'mini' bacteria present in seawater, and *Cafeteria* sp. grown on S-bacteria, had longer digestion times for S-FLB than did *Cafeteria* sp. grown on G-bacteria (Table 1). In our experiments, G bacteria had biovolume-specific C and N contents about half those of S bacteria. Slower digestion times for the smaller, but more nutrient-rich (in terms of unit biovolume) S bacteria apparently resulted in a more efficient digestion process, since the calculated carbon-based growth efficiency for *Cafeteria* sp. grown on S bacteria was about twice that for flagellates grown on G bacteria: 38.5% vs 21.5%. As the S bacteria used in these experiments were similar to natural marine bacterioplankton with respect to both cell size and per cell C and N contents, the implication is that feeding by protists on substrate-limited (starved) bacteria in natural microbial food webs will result in a greater proportion of ingested bacterial carbon being transferred to higher trophic levels, than would be the case if their bacterial prey were substrate replete and growing rapidly. In addition, more complete digestion of bacterial prey would be adaptive for bacterivore survival under conditions of prey scarcity, as obtains in most natural systems.

The case for differences in the digestion process of ingested small vs large bacterial cells is supported by results from investigations of the relation between the *in situ* activity of lysozyme, an enzyme which specifically degrades bacterial cell walls, at acid pH (i.e. in protist food vacuoles), and rate of bacterivory estimated via FLB uptake, determined for coastal and open ocean bacterivorous protists (González et al. 1993). Our results indicated that bacterivores grazing bacteria of 0.05 μm^3 average cell size in Pacific oligotrophic gyre water had much higher acid lysozyme activity rates for a comparable amount of bacterial biomass ingested compared to bacterivores grazing bacteria of 0.10 μm^3 average cell size in Oregon coastal waters (González et al. 1993). This may be related to the observation that the smaller the bacterial cell, the greater the ratio of cell wall material : total cell biovolume (Simon and Azam 1989).

Combining observations of size-selective grazing with our finding of differential digestion of large, growing vs small, starved bacteria, we hypothesize a feedback mechanism between phagotrophic flagellates and their bacterial prey. Under substrate replete conditions, flagellates would graze rapidly growing bacteria, especially dividing cells, at a faster rate, thus more effectively controlling bacterial abundance in bloom situations. However, flagellates grazing larger bacteria would exhibit 'sloppy feeding', in that ingested bacterial prey would be incompletely digested. This notion is supported by theoretical and empirical reports of less efficient digestion of food under 'luxury' abundances of prey by copepods and other invertebrates (Jumars et al. 1989), as well as by the finding of Nagata & Kirchman (1992) that up to 57% of prey biomass was released as macromolecules by flagellates feeding on large, cultured bacteria. On the other hand, lower clearance rates of starved, slower-growing bacteria might imply some degree of refuge for these bacteria from flagellate grazing (Chrzanowski & Simek 1990, González et al. 1990b, Sherr et al. 1992).

Grazing on motile and non-motile bacteria

FLB have been used to estimate grazing rates of bacteria by protists in a number of recent studies (e.g. Sherr et al. 1987, 1989, Bloem et al. 1988, Chrzanowski & Simek 1990, González et al. 1990b). The question of how well FLB uptake models grazing on live bacteria has still not been adequately addressed. Discrimination against heat-killed FLB in favor of natural bacteria has been proposed by several authors (Sherr et al. 1989, Landry et al. 1991, McManus & Okubo 1991).

In this study, we found that FLB are a reasonable surrogate for live non-motile bacteria, as clearance rates of LSB and FLB made from a non-motile bacterial strain (H1) were similar (Table 2). However, there were significantly higher clearance rates by flagellates of LSB compared to FLB for both a weakly motile bacterial strain (Y) and a highly motile strain (MB2A) (Table 2). Our results corroborate those of Monger & Landry (1992), who found significantly higher clearance rates by a marine flagellate for a motile bacterial strain compared to heat-killed cells of the same strain. Thus, use of heat-killed FLB could lead to underestimation of actual grazing rates if some fraction of bacterioplankton in a water sample were motile.

There is little information on the proportion of motile bacteria in natural microbial communities. Azam & Ammerman (1984) reported that a significant percent-

age of bacterioplankton in seawater samples were motile. Rheinheimer (1971) found a high percentage of motile strains in marine bacterial isolates. Both empirical results and theoretical considerations (Macnab & Koshland 1972, Azam & Ammerman 1984, Azam & Smith 1991, Lauffenburger 1991) suggest that motile bacteria could play an important role in aquatic ecosystems. Estimates of proportion of motile bacteria might vary depending on environmental conditions, such as substrate concentration, temperature, or physical turbulence (Macnab & Koshland 1972, Adler 1975, Lauffenburger 1991, Mitchell 1991, and pers. obs.). The issue of motility of *in situ* bacterioplankton is thus of critical importance for grazing assays using surrogate food particles. Our tentative conclusion is that grazing rates determined via FLB uptake could be underestimated by 50% or more if >1–10% of cells in a bacterioplankton assemblage exhibited motility.

There is a general consensus that the major consumers of suspended bacteria in most marine systems are phagotrophic flagellates (Azam et al. 1983, Bloem et al. 1988, Pace 1988, Sherr et al. 1989). Based on data presented in these reports, the average *in situ* ratio of bacterivory:bacterial cell production is 0.5 (range 0.2 to 1.0). However, if FLB uptake does seriously underestimate true grazing rates, bacterivory could be up to 2-fold greater than previously estimated, which would balance bacterivory and bacterial production in at least some of the cases reported.

Marine systems are highly heterogeneous; microzones of higher substrate abundance for heterotrophic bacteria have been postulated (Goldman 1974, Lehman & Scavia 1982, Mitchell et al. 1985, Jackson 1987, Jumars et al. 1989). Motile bacteria would appear to have an advantage compared to non-motile cells in their ability to move toward substrate-rich microzones (Azam & Ammerman 1984, Azam & Smith 1991, Lauffenburger 1991). However, the rate of bacterivory might also be increased in such microzones, which would tend to have higher bacterial concentrations than the bulk water column. Thus, if motile bacteria are subject to a greater degree of grazing mortality, as our and Monger & Landry's (1992) results suggest, their competitive advantage over non-motile bacteria might be reduced.

We conclude that feeding of phagotrophic flagellates on bacteria is significantly influenced by at least 2 qualitative aspects of prey cells: relative growth state and motility. In both cases, the implication is that flagellates clear more rapidly growing (substrate replete) cells (including motile cells capable of successfully exploiting substrate-rich microzones) faster than they do slower-growing (starved or non-motile) cells. This would serve to permit grazers to quickly control 'blooms' of bacteria and to diminish competition for resources between fast-growing and slow-growing bacteria within a microbial assemblage. However, more rapid clearance of larger, rapidly growing cells appears to be associated with less complete digestion of bacterial prey than is the case for smaller, slower-growing cells. If so, the normal situation for *in situ* microbial food webs in the sea, in which phagotrophic flagellates feed on C- and N-dense 'mini' bacteria, would optimize the transfer of bacterial biomass to higher trophic levels. Further work with *in situ* microbial assemblages is needed to confirm these hypothesized feedback mechanisms within microbial food webs.

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