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Rates of Microbenthic and Meiobenthic Bacterivory in a Temperate Muddy Tidal Flat Community

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Rates of bacterivory in micro- and meiobenthic species were determined by an improved technique in a muddy tidal flat community in Boston Harbor, Mass. The predominant grazers of bacteria were identified, and their rates of grazing were measured in the top 1 cm of the sediment. Grazing rates were measured by a fluorescence-labeled bacteria (FLB) technique. A mixture of two *Enterococcus* spp. isolates and two isolates of *Escherichia coli* were prepared as FLB, and they were added to intact sediment cores by replacing the pore water in the upper centimeter of the core. A standard FLB procedure was modified by filtering sediment dilutions onto cellulose membrane filters and processing the filters to render them optically transparent while preserving the physical integrity of the micro- and meiobenthic organisms. Thus, it was possible, on the same microscopic field, to switch from light microscopy for identification of grazers to epifluorescence microscopy for counting FLB present in the gut contents of the same grazers. The majority of benthic organisms present in these sediments consumed FLB, but their consumption rates varied widely. Two ciliate species, a *Prorodon* sp. and a *Chlamidodon* sp., and a nematode, a *Metoncholaimus* sp., consumed fluorescence-labeled coliforms at the highest rates, 126 to 169 FLB per individual per h. Other ciliates and nematodes, as well as microflagellates and harpacticoid copepods, consumed fluorescence-labeled coliforms at lower rates, 1.2 to 26 FLB per individual per h. Foraminiferans and gastrotriches did not contain FLB. Some ciliate grazers discriminated between enterococci and coliforms, consuming the rod-shaped fluorescence-labeled coliforms at 74- to 155-fold-higher rates than did the coccus-shaped fluorescence-labeled enterococci. Other ciliates did not select between fluorescence-labeled enterococci and fluorescence-labeled coliforms. The high rates of bacterivory by some ciliates and nematodes indicated intensive grazing. However, at their low extant densities, the grazers consumed only a small portion of the bacterial standing stock. Major bacterial grazers, e.g., microflagellates, ciliates, and nematodes, could potentially consume, per day, only 0.2, 0.1, and 0.03%, respectively, of the bacterial standing stock (7.5×10^8 bacteria per cm^3).

Secondary production by benthic bacteria may represent an important source of particulate organic carbon to benthic food webs (11, 26). Potential consumers of bacteria can be found in all three major groups of benthic animals, micro-, meio-, and macrobenthic (24), but the extent of benthic grazing on bacteria or of the potential partitioning of this food resource by benthic grazers is largely unknown.

Bacterivory by macrobenthic animals has received more attention than bacterivory by the microbenthic or meiobenthic animals. Macrobenthic animals do not appear to consume significant amounts of bacterial production, nor can bacteria alone meet the energy demands of the macrobenthos (6, 22, 24, 26). Therefore, various investigators have suggested that the primary grazers of benthic bacteria may be the smaller metazoans, for example, the meiobenthos, which is dominated by nematodes and harpacticoid copepods; and the protozoans, i.e., microbenthos, which are dominated by flagellates and ciliates (16, 37). However, the role of these groups as grazers of bacteria in the benthos has not been well established, and the conclusions from benthic grazing experiments are contradictory (20).

Only a few studies have been conducted on benthic grazing because, in part, the methodology for studying benthic bacterivory is fraught with difficulties (7–9, 13, 14, 28, 30, 46). Approaches that have been used to quantify micro- and meiobenthic grazing rates on benthic bacteria can be described as either indirect or direct. The indirect approach is based on inhibitors that selectively block bacterial growth and/or animal consumption of bacteria (30, 38, 40). The grazing rates on bacteria are calculated from the decrease in bacterial densities. The rates are dependent on the specificity of the inhibitors, but to varying degrees, some of the nontarget organisms are sensitive and some of the target organisms are resistant to the inhibitors. Thus, this approach can cause considerable uncertainty (46).

Bacterivory can be measured directly by amending sediments with bacteria labeled with either radioactive elements (19) or fluorochromes (23, 42). Alternatively, bacteria can be selectively labeled in situ with [^{3}H]thymidine on the predication that bacteria but not eukaryotic organisms become labeled with appropriate concentrations of [^{3}H]thymidine (17, 21, 30). This approach is attractive because it perturbs the community less than do the other methods and allows simultaneous measurement of bacterial production, but it generates other problems. The shortcomings include high rates of nonspecific binding of [^{3}H]thymidine, radiolabel accumulation by grazers bodies via nonfeeding processes, and intensive uptake of the label by enteric and epicuticular bacteria (or nongrazed bacteria) living inside and on the surface of potential grazers (30). Similar problems were encountered when ^{14}C-labeled tracers were used for measuring herbivory (3, 8, 28).

Fluorescence-labeled bacteria (FLB) have proved to be effective as food tracers in a number of recent studies in the water column (18). In the benthos, this technique has been

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used infrequently to quantify protozoan bacterivory (23, 33). We report here on a modified technique that uses FLB to measure bacterivory in the benthos. We used this technique to measure grazing rates on bacteria by protozoans, nematodes, and harpacticoid copepods in a muddy tidal flat community.

MATERIALS AND METHODS

Study site. The study was conducted on a benthic community at the low-tide level of a well-protected tidal flat in Savin Hill Cove, Boston Harbor, Mass., which has been described elsewhere (44). The sediment was composed of a fine mud; the salinity of overlying water was about 30‰, and the daytime temperature at the mud surface was 20°C. The experiment was conducted in May 1991.

Preparation of FLB. Four strains of enteric bacteria were used as model prey. *Enterococcus faecalis* ATCC 27274, *Enterococcus faecium* ATCC 19434, and two sewage treatment plant isolates of *Escherichia coli* were grown separately in tryptic soy broth (Difco, Detroit, Mich.) at 35°C. After a 24-h incubation, bacteria were centrifuged (10,000 × *g* for 10 min) and washed three times in 0.05 M Na_2HPO_4 in 0.85% NaCl solution. The suspension of *E. faecalis* was mixed with the suspension of *E. faecium*; the mean bacterial cell volume in the mixture was 0.07 μm^3. Similarly, the two *E. coli* strains were combined; the mean cell volume was 0.18 μm^3. Coliforms were stained for 2 h at 60°C with 2 mg of 5-([4,6-dichlorotriazin-2yl]amino)fluorescein (Sigma Chemical Co., St. Louis, Mo.) as described by Sherr et al. (42). The enterococcus mixture was stained with acridine orange (0.01%, final concentration) for 2 h at 60°C. After staining, bacteria were washed three times with the Na_2HPO_4-NaCl solution and resuspended in filter-sterilized seawater from the sample site. The seawater was filter sterilized by passing it through a 0.2-μm-pore-size Millipore (Bedford, Mass.) or Nuclepore (Pleasanton, Calif.) filter. To avoid clumps and obtain homogeneously dispersed cells, mixtures were filtered through 1.2-μm-pore-size Nuclepore filters and sonicated for 1 to 2 s. The density of fluorescence-labeled coliforms was adjusted to 4×10^8 cells per ml, and the density of fluorescence-labeled enterococci was adjusted to 3×10^8 cells/ml.

Grazing experiments. Intact sediments were obtained by hand coring with a 47-mm-inside-diameter polycarbonate corer (four replicates), and the cores were transported to the laboratory within 1 h of collection. The sediment was carefully extruded through the top of the corer, and the top 2-cm horizon was sliced off. The core slice (four replicates) was placed onto a scintered-glass Millipore filter unit with a 42-mm filtration surface diameter. The coliform mixture (6 ml) and enterococcus mixture (5 ml) were carefully layered on top of the sediments. Negative pressure was applied from beneath to replace the pore water in the sediment sections with the FLB mixtures. The fluorescence-labeled coliform and fluorescence-labeled enterococcus densities were 23 and 13%, respectively, of the total native bacteria density.

The FLB-inoculated sediments were incubated at 22°C for 1 h. During this incubation, subsamples were taken from each filter unit by coring the sediments with 8-mm-inside-diameter cores to a 1-cm depth. Thus, this work was restricted to the community inhabiting the upper 1 cm of the sediment. Subsamples were taken at 15, 30, 45, and 60 min of incubation from each of the four replicates. Subsamples consisted of two cores per time point for counting ciliates and the meiobenthos and a single core per time point for counting flagellates. Subsamples were immediately preserved with cold glutaraldehyde (2% [vol/vol], final concentration) and stored at 4°C in the dark prior to microscopic observations.

Microbenthic and meiobenthic organisms were extracted from preserved samples with Percoll (Sigma), a silica gel, according to Alongi (2) and Schwinghamer (39). Percoll (5 ml) was mixed with each sediment subsample (final volume of approximately 6 cm^3), and the samples were centrifuged at 500 × *g* for 10 min. The top layer, a mixture of Percoll and pore water containing benthic organisms, was carefully pipetted off the sediment, and fresh Percoll was added. The procedure was repeated three times with each sample, and the three fractions were combined. These mixtures were filtered through either 0.45- or 3-μm-pore-size cellulose nitrate Millipore filters, so that all organisms of interest extracted from the sample were collected onto a single filter.

Filters were covered with prewarmed (35°C) coverslips containing a drop of liquid agar (2% [vol/wt] solution). After a 5- to 15-s incubation in a freezer (−20°C), the agar hardened and coverslips could be removed. Thus, organisms collected on filters were protected by a thin layer of agar from being washed out during following treatments. The entire procedure is described in more detail by Montagnes and Lynn (32). Filters were further dehydrated in a standard series of progressive ethanol solutions (30, 50, 70, 96, 96, 100, and 100% [5 min in each]) and then immersed twice in xylene (5 min each time). This process results in almost completely transparent filters (40). The bodies of the meiobenthic grazers became similarly transparent. Treated filters were either embedded in Canadian balsam (permanent slides) or placed on a microscopic slide between two drops of low-fluorescence immersion oil and covered with coverslips (temporary slides).

In addition, several filters representing one subsample from each replicate were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) at a final concentration of 5 μg/ml before immersion in agar. These filters were used to examine and count microflagellates by epifluorescence microscopy on a Zeiss Standard microscope equipped with a Zeiss filter set (BP 365/10 exciter filter, FT 390 beam splitter, and LP 395 barrier filter; ×1,250 magnification). By scanning 100 to 150 microscopic fields per slide, we examined 20 to 35 total microflagellates per slide. Additional filters were stained with nigrosin (5) for ciliate identification according to Small and Lynn (45).

On all other slides, micro- and meiobenthic organisms were detected, identified, counted, and measured under bright-field phase-contrast microscopy on a Zeiss Standard microscope (×150 to ×1,250 magnification). For nematode identification, we used the guide by Platt and Warwick (35). Ingested FLB were counted in each benthic animal by epifluorescence microscopy (Zeiss filter set; BG 12 exciter filter, FI 450 beam splitter, and barrier filter 47; ×1,250 magnification) without disturbing the position of the slide. We examined 31 to 74 ciliates, 25 to 88 nematodes, 15 to 25 copepods, and 2 to 15 other metazoans on each filter. The four replicates used in the grazing experiments were sampled at four time points; thus, 16 filters were examined for flagellates, and 32 filters (two subsamples at each time point for each replicate) were examined for ciliates and meiobenthos.

The ingestion rates of native bacteria were then calculated by assuming that native bacteria were consumed in proportion to consumed FLB: $I = I_{FLB} \times [(N_{N.b.} + N_{FLB})/N_{N.b.}]$, where I is the grazing rate on native bacteria (bacteria per

TABLE 1. Benthic animal field densities, grazing rates on FLB, and calculated grazing rates on field bacteria

Organisms	Field density (individuals/cm^3 [SEM])	Ingestion rate	
		Fluorescence-labeled coliforms/individual (SEM)	Bacteria/individual/h
Microflagellates	1.1×10^4 (0.8×10^4)	1.2 (4.0)	5
Ciliates			
Prorodon sp.	18 (10)	169 (147)	704
Chlamidodon sp.	16 (22)	162 (128)	675
Others	50 (40)	26 (59)	108
Foraminiferans	<10	0	0
Nematodes			
Metoncholaimus sp.	14 (14)	126 (128)	525
Others	104 (58)	5 (31)	21
Harpacticoid copepods	42 (48)	10 (32)	42
Nauplii	18 (20)	8 (12)	33
Gastrotrichs	<10	0	0

individual grazer per hour), I_{FLB} is the grazing rate on FLB determined in the experiment (FLB consumed per individual grazer per hour), $N_{N.b.}$ is the field density of native bacteria (bacteria per cubic centimeter), and N_{FLB} is the concentration of added FLB (FLB per cubic centimeter).

For enumeration of native bacteria, four subsamples were taken at the beginning of incubation within 8-mm-inside diameter cores. Samples were fixed with 3 ml of formaldehyde (2%, final concentration), incubated with tetrasodium pyrophosphate (10 mM, final concentration), and sonicated three times for 10 s each time (47). After proper dilution, samples were stained with DAPI at a final concentration of 5 μg/ml (36). Each sample was filtered through a 0.2-μm-pore-size black Nuclepore filter and washed several times with filter-sterilized (0.2-μm-pore-size Nuclepore filters) seawater. Bacteria were detected by epifluorescence microscopy on a Zeiss Standard microscope equipped with a Zeiss DAPI filter set (see above). At least 200 cells per slide in at least 10 microscopic fields were counted.

The Student *t* statistic was used to calculate grazing rate confidence intervals, and Wilcoxon's two-sample test and binomial test were used to characterize differences between bacterial grazers.

RESULTS

Community structure. Sediment samples contained bacteria, microphytobenthic species (not studied), protozoans, nematodes, harpacticoid copepods and their nauplii, and gastrotrichs. The extant bacteria were mostly rod-shaped cells at a density of 7.5×10^8 cells per cm^3 (standard error, $\pm 2.2 \times 10^8$). Protozoans were represented by microflagellates, ciliates, and foraminiferans. Microflagellates, mostly small round cells 2 to 6 μm in diameter, outnumbered all other protozoans; their mean density was 1.1×10^4 cells per cm^3. The ciliated protozoan assemblage was relatively poor in both abundance and species diversity. The two species *Prorodon* sp. and *Chlamidodon* sp. represented 40% of the total ciliate density of only 84 individuals per cm^3. Foraminiferans were rare; their density was less than 10 individuals per cm^3.

Meiobenthic species composition was typical for intertidal communities. Nematodes were the predominant group, with a total density of 118 individuals per cm^3. Three species numerically dominated the nematodes: a *Metoncholaimus* sp., a *Comesoma* sp., and a *Monhystera* sp. Harpacticoid copepods represented the second most abundant metazoan group, with a total density of 42 individuals per cm^3. Other meiobenthic animals were rarer than nematodes and harpacticoids (total density, <20 individuals per cm^3; Table 1).

Bacterivory by the micro- and meiobenthos. Almost all animals examined ingested fluorescence-labeled bacteria, but the rates of FLB ingestion varied by over 2 orders of magnitude. The number of ingested fluorescence-labeled coliforms by a *Prorodon* sp., a *Metoncholaimus* sp., and other nematodes increased steadily with time (Fig. 1). In contrast, the number of fluorescence-labeled coliforms encountered in microflagellates, a *Chlamidodon* sp., unidentified ciliates, and nauplii did not maintain a steady increase beyond the 15-min incubation period. Therefore, ingestion rates of all animals were calculated from the number of fluorescence-labeled coliforms consumed in 15 min (Table 1).

Two ciliates, a *Prorodon* sp. and a *Chlamidodon* sp., and a nematode, a *Metoncholaimus* sp., had the highest coliform ingestion rates, 126 to 169 FLB per h. Other ciliates, as well as other nematodes, consumed either few fluorescence-labeled coliforms or none, with a resulting grazing rate of <5 to 26 FLB per h. Microflagellates as well as harpacticoid copepods and their nauplii also had low rates of FLB

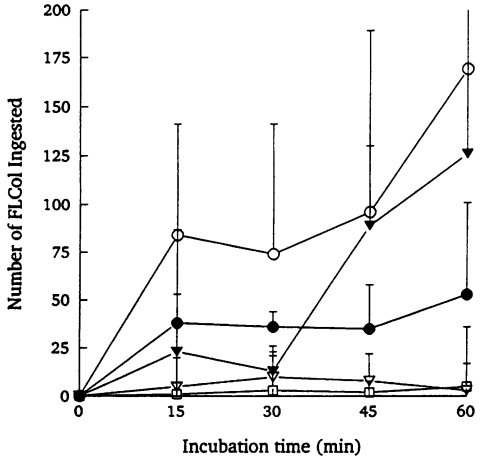

FIG. 1. Consumption of fluorescence-labeled coliforms (FLCol) by a *Prorodon* sp. (○), a *Chlamidodon* sp. (●), other ciliates (▽), a *Metoncholaimus* sp. (▼), and other nematodes (□) as a function of incubation time.

consumption, 1.2 to 10 fluorescence-labeled coliforms per h. On one occasion only, however, we observed a harpacticoid copepod specimen with the gut filled with thousands of fluorescence-labeled enterococci. Since it was the only specimen out of hundreds of harpacticoid copepods examined that contained FLB, we omitted that datum from further calculations. Other groups of benthic animals, foraminiferans and gastrotrichs, did not contain FLB.

Differences in ingestion rates were also distinguishable at the genus level (Table 1). Thus, the grazing rates on FLB were not only group specific but also at least genus specific; the two most abundant ciliate genera (represented probably by a single species each) consumed fluorescence-labeled coliforms at rates statistically higher ($P < 0.01$; Wilcoxon two-sample test) than the ingestion rates of other ciliates (Table 1). Similarly, a *Metoncholaimus* sp., a nematode, consumed fluorescence-labeled coliforms at a significantly higher rate ($P < 0.01$) than did other nematodes.

Benthic animals appeared to select between fluorescence-labeled enterococci and fluorescence-labeled coliforms. Unfortunately, acridine orange-labeled enterococci lost their fluorescence within 1 to 3 days following the completion of the experiment, so that the data comparing coliform consumption with enterococcus consumption were available only for ciliates, because they were processed and enumerated first. The binomial test indicates that the ratios of fluorescence-labeled coliforms to fluorescence-labeled enterococci in digestive vacuoles of the *Prorodon* sp. and the *Chlamidodon* sp. (155:1 and 74:1, respectively) were significantly different ($P < 0.01$) from the expected ratio (5:3). Some unidentified ciliates contained a 5:4 ratio of fluorescence-labeled coliforms to fluorescence-labeled enterococci, which was not significantly different from the expected ratio. Some ciliates, therefore, discriminated between enterococci and coliforms, with preference for rod-shaped coliforms, whereas others consumed different FLB without selection.

DISCUSSION

We measured bacterivory in the micro- and meiobenthos by using fluorescence-labeled bacteria as model food items. This technique is direct, since only FLB within the digestive vacuoles or guts of predators were counted. Much of the uncertainty inherent in grazing rates calculated by indirect approaches (14, 46) is averted. A major advantage of this approach is the ability to collect all potential grazers on one filter per sample for individual examination, eliminating the need for time-consuming hand sorting.

As the method is commonly used, it is not possible to identify all grazers with certainty on the same filter used to determine FLB consumption. Black polycarbonate filters are used to minimize background fluorescence, but they are not optically transparent. Thus, reliable identification is hampered and protozoans are typically lumped into a single group (33) or, at best, into two groups, microflagellates and ciliates (23). The use of cellulose membrane filters cleared in xylene allows, in a single microscopic field, simultaneous enumeration of consumed FLB by epifluorescence microscopy and identification of the grazer. Xylene also clears the bodies of metazoans so that they become almost transparent; therefore, FLB in grazer guts can be counted directly with fluorescence microscopy. An additional advantage of cellulose membranes is that distinction between internal FLB and FLB absorbed on the outer surface of the grazer's body is much easier than differentiation on polycarbonate filters.

The modified technique yielded considerable information about benthic grazing in the muddy tidal flat, but none of the techniques available are without flaws. Two aspects of the FLB technique could lead to bias in the determination of grazing rates. First, the addition of FLB to the sediment can disrupt the community and influence animal feeding rates and behavior in an unpredictable manner (9, 13), although it was not obvious whether, as with a fluid label, this technique provided a uniform distribution of FLB in the sediment. If, in fact, FLB were not distributed evenly along the vertical gradient and were concentrated within a few upper millimeters of the sediment, then we selected for grazers at the water-sediment interface. Of the various approaches to amending the sediment, the pore water replacement technique is one of the least disruptive (9, 13). Second, discrimination of prey on the basis of size or type by grazers can influence the estimate of total bacterivory. Influence by prey size and shape in food selection has been well documented at least in planktonic protozoans (10, 18). A possible selection for free bacteria rather than attached bacteria by water column grazers was shown by Albright et al. (1). Therefore, the choice of bacteria for FLB preparation may be crucial. We used allochthonous coccus- and rod-shaped FLB to account for size and shape variations in prey, although most of the data obtained are based on consumption of coliforms only. However, rod-shaped bacteria were predominant in the benthos.

Further, the FLB-based technique, i.e., the direct technique that employs microscopic observation of the contents of digestive vacuoles or animal guts, necessitated the use of glutaraldehyde to preserve samples since it does not provoke the expulsion of the digestive vacuoles (4). Glutaraldehyde, however, has been shown to cause the loss of a third of the ciliates in samples stored for two months (34). If protozoans are enumerated from stored samples, their actual abundances may be underestimated.

High background fluorescence of cellulose filters makes visualization of the smallest protozoans, the microflagellates, more difficult since the filters must be stained with a fluorescent stain for adequate visualization. Therefore, the original technique of Sherr et al. (42) is more appropriate for studying flagellate bacterivory.

Although it was technically difficult to do so, FLB could still be counted in small protozoans to provide an estimate of microflagellate grazing rates on FLB (Table 1). Our estimate is on the low end of the range of rates reported in the literature for planktonic species (2 to 363 bacteria per flagellate per h [4, 12, 25, 27, 41]) but comparable to the rate of benthic bacterivory (2 bacteria per flagellate per h) estimated by Kemp (24). Unless we severely underestimated the flagellate feeding rate, the density of microflagellates in the community that we sampled was probably too low to affect bacterial abundances. The flagellate-to-bacterium ratio in the muddy tidal flat was 1:70,000. Kemp (24) suggested that a ratio greater than 1:1,000 is necessary to affect bacterial abundance. We estimate that the entire microflagellate assemblage could potentially consume only 0.2% of the bacterial standing stock per day.

In contrast to the microflagellates, the ciliates were voracious grazers of bacteria (Table 1). At least some ciliate species, especially in planktonic communities, are intensive bacterivores (18, 43, 42). High ciliate grazing rates on sediment bacteria (37 to 600 bacteria per ciliate per h) were reported in a few benthic studies that focused on ciliate grazing (15, 23). Despite high grazing rates by individual ciliates, their role in balancing bacterial production in the

muddy tidal flat was probably negligible. The total ciliate abundance at the time of the experiment was low (Table 1), and the entire ciliate assemblage could potentially consume only 0.1% of the bacterial standing stock per day.

Similarly, grazing by the meiobenthic species did not appear to have an effect on bacterial dynamics. Most of the meiobenthic species contained FLB in their guts, but only one nematode species, the *Metoncholaimus* sp., consumed FLB at high rates (Table 1). The ability of both nematodes and harpacticoid copepods to consume sediment bacteria in a number of habitats is well documented (9, 29, 30, 37). At extant densities in the tidal flat sediments, they could potentially account for the consumption of only 0.03% of the bacterial standing stock per day. Other nematode species and the harpacticoid copepods essentially disregarded bacterial prey. Taking a bacterial turnover value of 10 days from previous studies (22, 24), bacterivorous ciliates and meiobenthic species must be 10^4 individuals per cm^3 to balance the bacterial production by our estimated grazing rates. This is an unrealistically high density. Rather, our results support the findings of Montagna et al. (31), who reported an absence of spatial correlations between bacterial and meiofauna abundances. The ability of the meiobenthos to balance bacterial production has been shown (29), but in that case the predominant grazing pressure was attributed to polychaetes. Our data provide additional evidence that on muddy tidal flats, the micro- and meiobenthos may not influence bacterial numbers by grazing. If macrofaunal feeding has also only a marginal effect on bacterial abundances (22), the major part of the bacterial production in the muddy tidal flat sediments might not be directly influenced by grazing at all.

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