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Differential Rates of Digestion of Bacteria by Freshwater and Marine Phagotrophic Protozoa

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Differential decreases over time of two bacterial species, *Escherichia coli* and *Enterococcus faecalis*, in a freshwater and a marine ecosystem were observed and explained by a differential rate of digestion of these bacteria by phagotrophic flagellates and ciliates. For this purpose, fluorescence-labeled bacteria (FLB) were used and prepared from the two species cited above. The number of FLB was observed for 5 days in fresh and marine waters in the presence or absence (0.2-μm-pore-size-filtered water) of natural microbiota. These experiments showed a longer persistence of *Enterococcus faecalis* FLB as opposed to *Escherichia coli* FLB in the presence of natural microbiota. Removal of FLB was due to protozoan grazing because no decrease of FLB number was observed in the absence of natural microbiota. In short-term (about 40 min) ingestion experiments, we found similar clearance rates of *Escherichia coli* and *Enterococcus faecalis* FLB by assemblages of flagellates from the freshwater and the marine ecosystem and by cultured assemblages of ciliates from the marine ecosystem. Clearance rates of *Enterococcus faecalis* FLB were greater than those of *Escherichia coli* FLB for assemblages of ciliates from the freshwater ecosystem. Comparison of rates of ingestion and digestion of FLB by protozoa showed that *Escherichia coli* FLB were digested and ingested at similar rates. However, *Enterococcus faecalis* FLB were digested slower than they were ingested. These results suggest that a longer persistence of *Enterococcus faecalis* as opposed to *Escherichia coli* can be explained by a differential digestion by flagellates and ciliates in aquatic ecosystems. Moreover, rates of ingestion and digestion were strongly correlated for both FLB types.

The ecological role of heterotrophic protozoa, both flagellates and ciliates, has attracted increased attention in recent years as a result of their importance as bacterial consumers in aquatic environments (3, 12, 13, 21, 29, 36, 40, 40a). Methods most frequently used to quantify protozoan grazing upon bacteria involve checking either changes in abundance of bacteria or bacterial analogs over a period ranging from hours to days (11, 25, 27, 28, 48) or rates of incorporation of bacterial analogs (mainly fluorescent microspheres or fluorescence-labeled bacteria [FLB]) by protozoa (7, 29, 40).

Removal of specific bacterial species in natural aquatic ecosystems has been a focus of interest for many years. Several researchers (4, 11, 16, 17, 27, 28) studying the evolution of culturable enteric bacteria in natural waters when natural microbiota were present found decreases of these bacterial species during their experiments. Most of these investigators (4, 11, 16, 27, 28) reported that protozoan grazing was the major factor responsible for bacteria decreases, since decreases in the number of culturable bacteria were accompanied by increases in protozoan numbers. Moreover, some authors (16, 17; I. Barcina, J. M. González, J. Iriberri, and L. Egea, submitted for publication) reported differential persistence of enteric bacteria in natural waters when natural microbiota were present.

According to Mitchell et al. (30), bacteriovorous protozoa do not necessarily consume bacteria of different species with equal efficiency. Different growth rates of protozoa grazing on different bacterial prey have been demonstrated for cultures of both ciliates (10, 46) and flagellates (30, 39).

FLB (40) have been accepted as a suitable method to determine rates of ingestion of bacteria by protozoa (1, 6, 40a), and Sherr et al. (41) have already demonstrated that FLB are a useful tool for the analysis of rates of bacteria digestion in protozoa since FLB support growth of flagellates and ciliates at rates similar to those supported by unstained bacteria.

The aim of this study was to show the role of protozoa in the differential consumption of bacterial species from aquatic ecosystems. To reach this objective, we first corroborated that the FLB made from the two bacterial species proposed for this study, *Escherichia coli* and *Enterococcus faecalis*, were in fact differentially removed by protozoa from natural waters. Second, to compare consumption of these monospecific FLB by protozoa, we determined rates of ingestion and digestion of FLB by flagellates and ciliates in a freshwater and a marine ecosystem. It is concluded that, at least in this case, the differential rates of digestion of bacteria by protozoa could explain the observed differences in bacterial mortality owing to protozoan predation between some bacterial species.

MATERIALS AND METHODS

Sampling sites were the Butrón River (Spain) and La Salvaje beach (Spain). All samples were collected just beneath the surface, and those from the marine ecosystem were at 500 m from the coast.

Preparation of FLB. FLB stained with 5-([4,6-dichlorotriazin-2-yl]amino) fluorescein were prepared as described by Sherr et al. (40) from two strains of the American Type Culture Collection (Rockville, Md.): a gram-negative bacterium, *Escherichia coli* ATCC 11775, and a gram-positive bacterium, *Enterococcus faecalis* ATCC 19433. These bacteria were grown overnight in nutrient broth and harvested by centrifugation before FLB preparation.

Both monospecific FLB had a similar size, 0.32 ± 0.13

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μm^3 for *Escherichia coli* FLB and 0.32 ± 0.10 μm^3 for *Enterococcus faecalis* FLB.

Long-term experiments. Long-term experiments were done five times to check removal of *Escherichia coli* and *Enterococcus faecalis* FLB over a period of 5 days. An inoculum of about 10^7 FLB ml^{-1} was added to water samples with and without natural microbiota and then incubated in the dark with shaking at room temperature. Natural samples contained about 1×10^6 to 5×10^6 natural bacteria ml^{-1}. Samples without natural microbiota were obtained by filtering natural waters through 0.2-μm-pore-size filters (Millipore Corp., Bedford, Mass.). Subsamples were taken periodically and preserved with buffered Formalin (2% final concentration). FLB were enumerated by using unstained 0.2-μm-pore-size polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.) and epifluorescence microscopy (40).

Short-term experiments. Short-term experiments were done to compare both uptake, or ingestion, of FLB by protozoa and disappearance, or digestion, of FLB in protozoan cells.

The experimental design followed that described by Sherr et al. (41). Briefly, after the addition of FLB to the sample, a linear uptake of FLB by protozoa is observed. This linear uptake of FLB generally ceases after 10 min to 1 h (depending on samples and experimental conditions), after which uptake is equilibrated with disappearance of FLB in the protozoa. A 10-fold dilution with natural water containing the same concentration of unlabeled bacteria decreases uptake to 1/10th of the initial rate. Then it is possible to observe the decrease of FLB within the protozoan cells over time, which corresponds to rate of egestion-digestion of FLB by protozoa. After dilution, a linear decrease in the numbers of FLB per cell is generally observed.

These experiments were conducted in 400-ml Whirl-pak bags presoaked in 10% HCl and copiously rinsed with deionized water. Dilution controls were set up for each experiment. The controls consisted of protozoan assemblages plus a concentration of FLB equal to that reached in noncontrol bags after dilution.

Flagellate ingestion-digestion experiments were conducted with natural populations of flagellates from fresh and marine waters. For flagellates, four short-term experiments were done with each FLB type (*Escherichia coli* and *Enterococcus faecalis*). More than 95% of these colorless flagellates ranged from 2 to 8 μm in diameter. Four 400-ml Whirl-pak bags were prepared for each ingestion-digestion experiment. Two experimental bags received 200 ml of natural water, and the two others, used for the dilution controls, received 50 ml. The bags were then placed in the dark in a bath at room temperature (about 17 to 19°C) for 30 min to allow the protozoa to recover from handling shock. FLB were inoculated into the experimental bags at concentrations of from 5 to 50% of total bacteria.

A time course started after the addition of FLB. Samples of 10 ml were taken over 1 to 2 h. Samples were fixed by the Lugol-Formalin decoloration technique (41), which prevents both preservation-induced egestion of FLB by protozoa and lysis of ciliates (40a, 41).

Immediately after the initial 1 to 2-h time course of sampling, 50 ml of water used in the uptake experiments was diluted with 450 ml of 0.2-μm-pore-size-filtered natural water containing appropriate numbers of bacteria to approximate the total concentration of bacteria initially present in the water sample (41). The dilution control bags containing 50 ml of water sample were also diluted as above, and then FLB were added to give the same concentration as in the diluted, experimental bags. Samples of 50 ml were taken over a second time course of 1 h from both experimental and control bags.

The disappearance rate of FLB per cell was corrected for FLB uptake after dilution. Slopes of increase and decrease of FLB per cell were determined via regression analysis ($n \geq 4$) for each ingestion-digestion experiment and compared by the F test for the difference between the absolute regression coefficients (43). Per cell clearance rates were calculated by dividing the average number of FLB $cell^{-1}$ by the concentration of FLB in the experimental bags. Clearance rates were compared by the Student t test (43).

Ciliate ingestion-digestion experiments were done with cultured assemblages of freshwater and marine ciliates as a consequence of the low number of ciliates present in the two studied ecosystems. For ciliates, four and five ingestion-digestion experiments were conducted with *Escherichia coli* FLB and *Enterococcus faecalis* FLB, respectively. The major group of ciliates present in these ecosystems was tentatively classified as members of the order *Scuticociliatida*. Ciliate assemblages were obtained adding 0.1% (wt/vol) yeast extract to natural water samples (26) and incubating at room temperature in the dark without shaking. After 2 to 3 days, high numbers of ciliates were detected and the experiment was started. The rest of the experimental protocol was the same as for flagellates.

Preserved protozoan samples were stained with 4′,6-diamidino-2-phenyl-indole (35) and filtered onto 0.8-μm-pore-size (for flagellates) and 3-μm-pore-size (for ciliates) black polycarbonate filters (Nuclepore Corp.). A minimum of 30 protozoa were inspected for each time period to determine the average number of FLB per cell. Flagellates were observed at a magnification of ×1,250 and ciliates at ×500; FLB within each protozoan were enumerated at ×1,250. The numbers of FLB in each bag were determined as described above. The total numbers of bacteria were determined by the acridine orange direct count method (22).

RESULTS

Long-term experiments. Figure 1 shows two examples of the long-term experiments done in fresh and marine waters with and without natural microbiota. In the absence of natural microbiota, no decrease of FLB number was observed, indicating that FLB remain fluorescent after at least 5 days. However, in the presence of natural microbiota, *Escherichia coli* FLB decreased down to 0.02% of the initial number of FLB in fresh water and to 0.7% in marine water, whereas *Enterococcus faecalis* FLB decreased only down to 13.7 and 25.9% (in fresh and marine water, respectively) of the initial FLB number after 5 days.

Short-term experiments. Figures 2 and 3 show some examples of the ingestion-digestion experiments. The correlation coefficient for the linear regression fits to the data was >0.95 ($n \geq 4$) in all cases for both ingestion and digestion time courses. These short-term experiments show that both *Escherichia coli* FLB and *Enterococcus faecalis* FLB were actively grazed by flagellates and ciliates from freshwater and seawater.

Owing to the fact that ingestion rates (FLB $cell^{-1}$ $minute^{-1}$) are affected by variations in the number of FLB in the bags, relative clearance rates (nanoliters $cell^{-1}$ $hour^{-1}$) can be more useful to compare uptake of *Escherichia coli* and *Enterococcus faecalis* FLB by protozoa (Table 1). Thus, we detected no differences between clearance rates of *Escherichia coli* FLB and *Enterococcus faecalis* FLB by natural

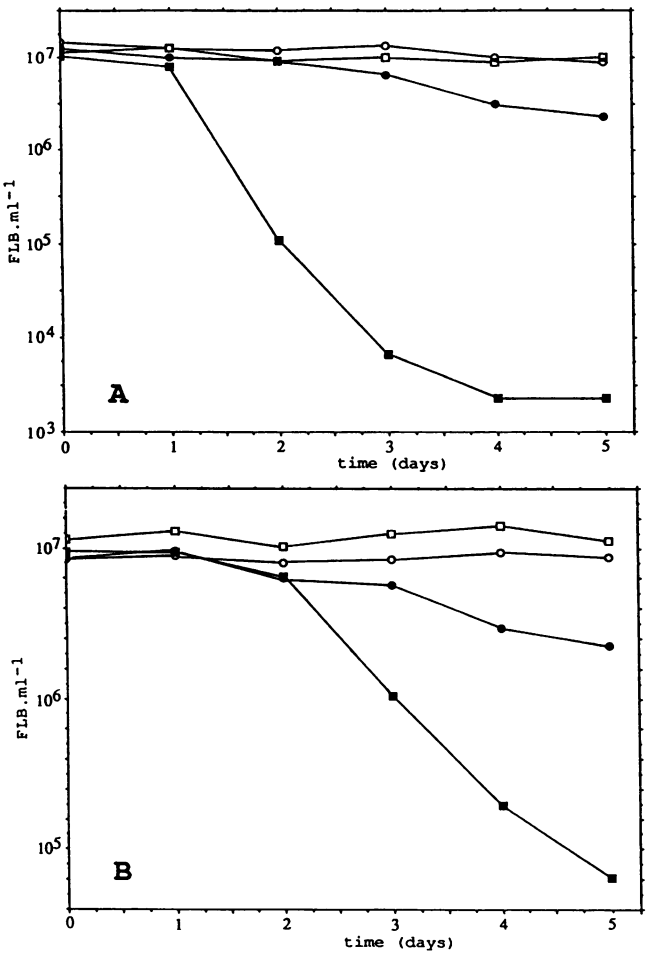

FIG. 1. Representative long-term experiments done in fresh (A) and marine (B) waters with *Escherichia coli* FLB in the presence (■) and absence (□) of natural microbiota and with *Enterococcus faecalis* FLB in the presence (●) and absence (○) of natural microbiota.

populations of flagellates from fresh and marine waters. There were also no differences for mixed species assemblages of marine ciliates, although for mixed species assemblages of freshwater ciliates significant differences ($P < 0.01$) between clearance rates of *Escherichia coli* FLB and *Enterococcus faecalis* FLB were observed. Clearance rates of *Enterococcus faecalis* FLB by freshwater ciliates were from two to three times greater than those for *Escherichia coli* FLB.

Comparing absolute rates of ingestion and digestion for each experiment, no significant differences were found for *Escherichia coli* FLB for either flagellates or ciliates in freshwater and seawater (Fig. 2 and 3). However, when rates of ingestion and digestion for *Enterococcus faecalis* FLB were compared, there were significant differences ($P < 0.001$) in all cases. Rates of ingestion of *Enterococcus faecalis* FLB were greater than absolute rates of *Enterococcus faecalis* FLB digestion. Calculated rates of ingestion and digestion of both FLB types for the flagellate and ciliate experiments are shown in Tables 2 and 3, respectively.

Highly significant relationships ($P < 0.001$) between rates of ingestion and rates of digestion for both *Escherichia coli* FLB ($r = 0.99$, $n = 9$) and *Enterococcus faecalis* FLB ($r = 0.98$, $n = 8$) were observed. Figure 4 shows these plots and the obtained linear regressions. Note that both slopes were almost equal, 0.964 and 0.961 for *Escherichia coli* and *Enterococcus faecalis*, respectively. Analysis of variance regression analysis (43) revealed that variations of ingestion rates could explain 99.99% of variations of digestion rates for a single bacterial species.

DISCUSSION

The decrease in the numbers of culturable enteric bacteria in natural waters when natural microbiota was present has been used to show that bacteriovorous protozoa are the major factor responsible for the elimination of specific bacterial species from natural aquatic ecosystems (4, 11, 16, 17, 27, 28; Barcina et al., submitted). However, the decrease in the numbers of culturable bacteria could also be either the result of a loss of culturability or a decrease due to predatory agents besides protozoa, e.g., bacteriophages (5). With respect to culturability, several workers (19, 34, 38) have cited a decrease in the numbers of culturable enteric bacteria in experiments done in 0.2-μm-pore-size-filtered natural water. Use of FLB allowed us to examine the elimination of specific bacterial species by protozoa in natural waters, since FLB decrease is estimated by direct counts, thus avoiding the problem of culturability of bacteria.

Predatory microbes, i.e., *Bdellovibrio* sp. (20, 28, 31, 45), and bacteriophages (5, 8, 14, 37) have also been proposed as a possible cause of bacterial mortality in natural waters, although their quantitative importance remains controversial (33, 40a, 42). However, since these predatory microbes appear to lyse only living bacteria (8, 23, 42) and FLB are heat-killed bacteria, the use of FLB would avoid the possible effects of these predatory microbes on tested bacteria. Moreover, controls done in the absence of natural microbiota showed that neither spontaneous lysis of FLB nor loss of fluorescence occurred during our 5-day experiments. Thus, decrease in the numbers of FLB was the result of protozoan predation only. This represents a useful approach to the study of removal of specific bacteria from the ecosystems resulting from protozoan grazing.

Differential persistence between bacterial species in aquatic ecosystems has been shown (16, 17, 24; Barcina et al., submitted). Because removal of specific bacteria from the aquatic ecosystems can be attributed to protozoan predation, those differences in persistence of specific bacteria when natural microbiota was present should also be a consequence of grazing by protozoa. In our study, the FLB technique also permitted us to detect differences in persistence of specific bacteria in natural aquatic ecosystems (Fig. 1) which revealed that both flagellates and ciliates were able to differentiate between monospecific FLB of a similar size, as they do with live bacteria (10, 30, 39, 46).

Differential utilization of bacteria by protozoa has been shown by different growth responses of ciliates (10, 46) and flagellates (30, 39) to different prey bacteria. This has been explained (30, 39, 46) in terms of differential food quality of different bacterial species. Some (10, 46) have pointed out that, in general, gram-positive bacteria are inferior food for protozoa. In this respect, the concept of food specialization has been proposed for ciliates (46), which would explain why protozoan growth is lower with gram-positive bacteria as prey (10, 46). It may be that protozoa are not well adapted to consume gram-positive bacteria owing to the low abundance of endemic gram-positive bacteria in natural aquatic ecosystems (49).

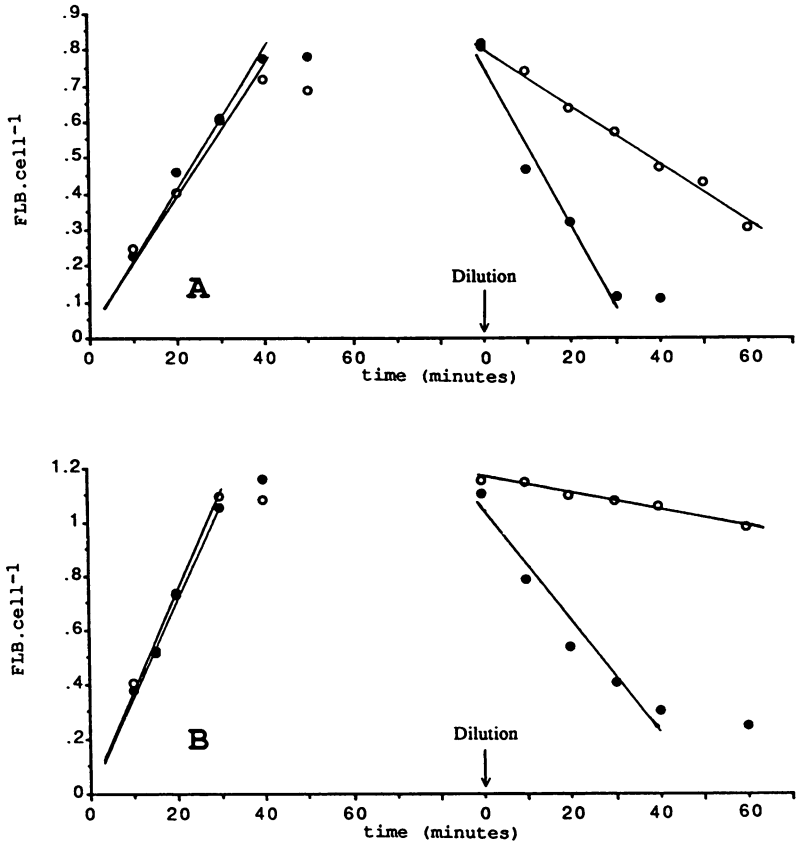

FIG. 2. Representative short-term experiments done with *Escherichia coli* FLB (●) and *Enterococcus faecalis* FLB (○) for natural populations of flagellates from fresh (A) and marine (B) waters.

However, results from our short-term experiments showed that clearance rates of monospecific FLB by flagellates and ciliates (Table 1) could not explain the longer persistence of *Enterococcus faecalis* FLB as opposed to *Escherichia coli* FLB. We found that mixed species assemblages of ciliates from fresh water cleared *Enterococcus faecalis* FLB faster than they did *Escherichia coli* FLB, although natural populations of flagellates from fresh water and seawater and mixed species assemblages of ciliates from seawater ingested both monospecific FLB at similar rates.

We also found that both for flagellates (Fig. 2; Table 2) and for ciliates (Fig. 3; Table 3), *Escherichia coli* FLB were ingested and digested at similar rates but *Enterococcus faecalis* FLB were digested slower than they were ingested. Taylor and Berger (46), studying several species of ciliates and bacterial prey, pointed out that bacteria may be ingested but not digested. This could be the case for *Enterococcus faecalis* FLB which were ingested but then digested with difficulty. In this respect, the contributions of King et al. (24) should be noted. They showed that ingested bacteria can resist digestion by protozoa and demonstrated the differential survival of bacteria within protozoa during chlorination. Our results agree with those obtained by King et al. (24) since differential digestion of different bacterial species by protozoa was observed in both studies, that of King et al. (24) with laboratory cultures of ciliates and the present one with mixed species assemblages of flagellates and ciliates. Thus, differential digestion in microbial food webs and public health should be taken into account.

After the time needed for processing of food vacuoles by protozoa (15), these undigested bacteria could be expelled. Thus, some of the bacterial prey would not be removed from the aquatic ecosystems but would be available for reingestion (24, 32). Nilsson (32) speculated that longer digestion times seen for some bacteria in *Tetrahymena* sp. could be the result of thicker cell walls, and gram-positive bacteria, such as *Enterococcus faecalis*, are characterized by thick cell walls (44). Thus, longer digestion times of FLB or unlabeled bacteria by protozoa could explain the differential

TABLE 1. Clearance rates of natural populations of flagellates and mixed assemblages of ciliates from freshwater and seawater based on uptake of *Escherichia coli* FLB and *Enterococcus faecalis* FLB[a]

Protozoan type	Ecosystem	Clearance rate (nl cell^{-1} h^{-1})[a] of:		P^b
		Escherichia coli FLB	*Enterococcus faecalis* FLB	
Flagellates	Freshwater	2.616 ± 0.600	2.467 ± 0.397	NS
		1.269 ± 0.093	1.374 ± 0.168	NS
	Marine	2.820 ± 0.094	2.785 ± 0.181	NS
		0.633 ± 0.029	0.691 ± 0.081	NS
Ciliates	Freshwater	4.848 ± 0.589	9.222 ± 0.641	<0.001
		1.614 ± 0.394	5.722 ± 1.022	<0.01
		9.788 ± 2.694		
	Marine	238.1 ± 43.26	268.0 ± 43.97	NS
		83.17 ± 12.71	83.87 ± 10.89	NS

[a] Mean value ± 1 standard deviation.
[b] Levels of significant difference (P) between clearance rates for both FLB types. NS, No significant difference.

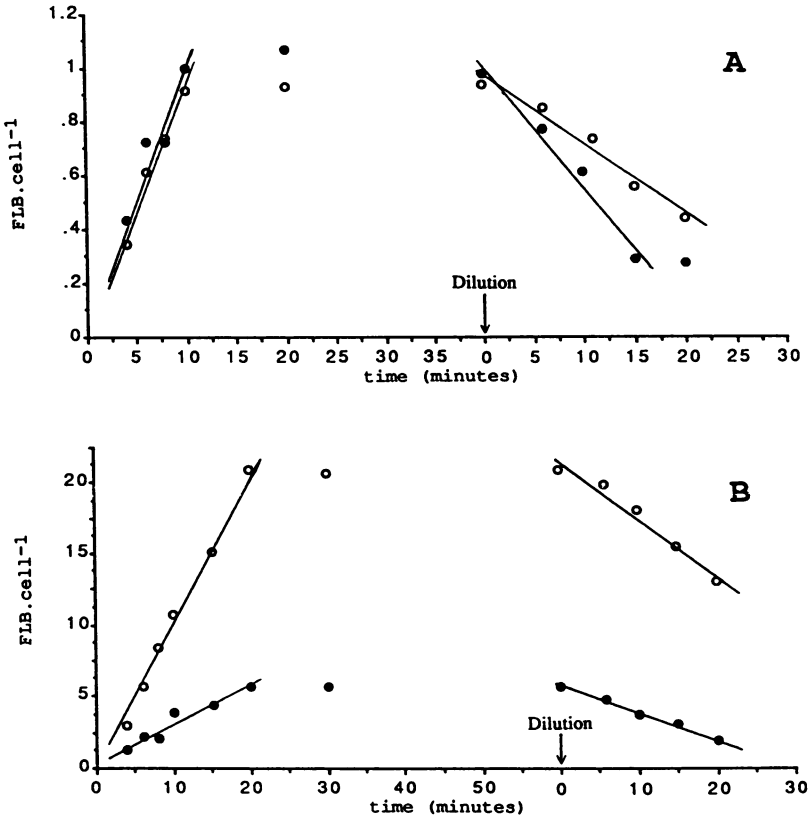

FIG. 3. Representative short-term experiments done with *Escherichia coli* FLB (●) and *Enterococcus faecalis* FLB (○) for mixed species assemblages of ciliates from fresh (A) and marine (B) waters.

elimination of specific FLB or bacterial species from the aquatic ecosystem.

Our estimates of ingestion and digestion rates by protozoa agree with those from other investigators. Sherr et al. (41) reported similar rates of ingestion and digestion for mixed species assemblages of flagellates and cultures of ciliates grazing on FLB made from cultures of natural bacterial assemblages. There was a significant linear relationship ($P < 0.001$) between rates of ingestion and digestion from their data ($y = -0.038 + 0.958x$). In our experiments, similar

TABLE 2. Comparison of rates of ingestion and digestion of *Escherichia coli* FLB and *Enterococcus faecalis* FLB by natural populations of flagellates from a freshwater and a marine ecosystem

Ecosystem	FLB type	Ingestion rate (FLB cell^{-1} min^{-1})	Digestion rate (FLB cell^{-1} min^{-1})	P^a
Freshwater	*Escherichia coli*	0.029	−0.032	NS
	Enterococcus faecalis	0.031	−0.006	<0.001
	Escherichia coli	0.019	−0.024	NS
	Enterococcus faecalis	0.018	−0.010	<0.001
Marine	*Escherichia coli*	0.035	−0.028	NS
	Enterococcus faecalis	0.036	−0.008	<0.001
	Escherichia coli	0.008	−0.009	NS
	Enterococcus faecalis	0.007	−0.004	<0.001

[a] Levels of significant differences between ingestion and digestion rates. NS, No significant difference.

TABLE 3. Comparison of rates of ingestion and digestion of *Escherichia coli* FLB and *Enterococcus faecalis* FLB by mixed assemblages of ciliates from fresh and marine waters

Ecosystem	FLB type	Ingestion rate (FLB cell^{-1} min^{-1})	Digestion rate (FLB cell^{-1} min^{-1})	P^a
Freshwater	*Escherichia coli*	0.097	−0.075	NS
	Enterococcus faecalis	0.093	−0.036	<0.001
	Escherichia coli	1.653	−1.596	NS
	Escherichia coli	0.008	−0.008	NS
	Enterococcus faecalis	0.022	−0.014	<0.001
Marine	*Escherichia coli*	5.217	−4.502	NS
	Enterococcus faecalis	7.232	−1.902	<0.001
	Escherichia coli	0.284	−0.249	NS
	Enterococcus faecalis	1.068	−0.619	<0.001

[a] Levels of significant differences. NS, No significant difference.

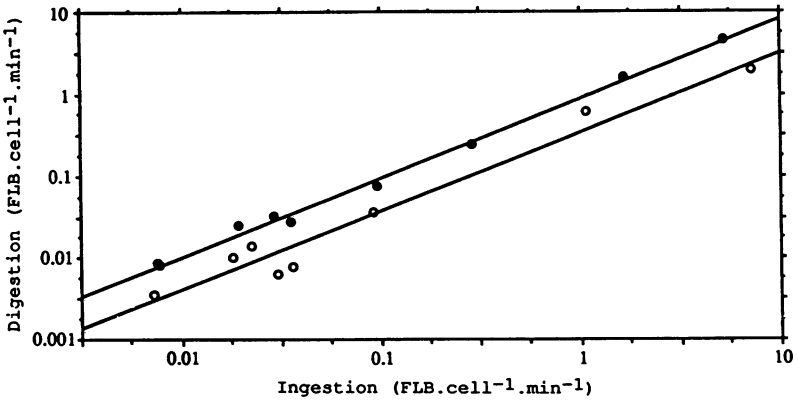

FIG. 4. Relationship between rates of ingestion and rates of digestion of *Escherichia coli* FLB (●) and *Enterococcus faecalis* FLB (○). Linear regressions are $y = -0.053 + 0.964x$ for *Escherichia coli* FLB and $y = -0.456 + 0.961x$ for *Enterococcus faecalis* FLB.

results were obtained for natural populations of flagellates and mixed species assemblages of ciliates grazing on *Escherichia coli* FLB, but our grazing experiments with *Enterococcus faecalis* FLB showed slower digestion than ingestion rates. Nevertheless, we found significant linear relationships ($P < 0.001$) between rates of ingestion and digestion by protozoa of both *Escherichia coli* FLB and *Enterococcus faecalis* FLB (Fig. 4). These results indicate that rates of digestion are mainly affected (i) by rates of ingestion of bacteria by protozoa and (ii) by the nature of the ingested food, that is, by the bacterial species. However, clearance rates per protozoan cell or uptake of bacteria by protozoa can undoubtedly be affected by several parameters, such as protozoan type and growth state (12, 13, 41), temperature (9, 41), bacterial abundance (12, 13, 41), and size and nature of bacterial prey (2, 18, 47).

From the present study, it is concluded that differential digestion rates of ingested bacteria by protozoa is one explanation for the observation that bacterial species of similar size can be differentially removed from the aquatic ecosystems by protozoa. Nevertheless, some studies (2, 13, 18, 47) have reported that bacteria can be ingested at different rates by protozoa, but in these cases the different size of bacterial prey was the variable parameter.

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