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Characterization of Culturability, Protistan Grazing, and Death of Enteric Bacteria in Aquatic Ecosystems

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Nonstained bacteria (NSB), rhodamine-stained bacteria (RSB), and fluorescence-labeled bacteria (FLB) were prepared from two enteric bacterial species, *Escherichia coli* and *Enterococcus faecalis*. Counts of CFU of NSB and RSB and total numbers of RSB and FLB were monitored over time, both in the presence and in the absence of natural microbiota. In the presence of natural microbiota, no differences were observed between CFU counts of NSB and RSB, but RSB total numbers were 1 to 4 orders of magnitude higher than CFU numbers. Therefore, the use of standard bacteriological media causes an important underestimation of the total number of enteric bacteria. In the absence of natural microbiota, the total numbers of NSB, RSB, and FLB remained constant over time. These results showed that RSB are a reliable indicator of the decay in both the total number and the CFU of enteric bacteria in natural water samples. By using RSB, enteric bacteria were classified as culturable cells, nonculturable cells (or somnicells), and dead cells in the presence of natural microbiota. In the presence of natural microbiota, differences between RSB and FLB direct counts were detected for *E. coli*, but not for *E. faecalis*. These differences were explained by size-selective grazing. Thus, protistan grazing was found to be the main cause of the decrease in total numbers of enteric bacteria in our experiments.

Considerable interest has been shown about parameters that affect the enumeration and fate of fecal coliforms and streptococci, commonly used as indicators of fecal pollution in natural and drinking waters (5, 11, 13). Standard bacteriological methods, based on plate counts, have been increasingly criticized as inefficient estimators of the total number of bacteria (5, 11, 19, 35); large differences have been reported between plate and total direct counts (5, 11, 19, 35). As such, cells from any bacterial population can be classified as belonging to one of two fundamental types, culturable cells and nonculturable cells (or somnicells) (36). Culturable cells are able to form colonies on standard culture media; nonculturable cells can be enumerated only by direct counts (4, 5, 11, 35, 36), but are not currently detected by standard analysis. Moreover, several authors (5, 19, 35, 36) have shown that nonculturable bacteria can be metabolically active and that nonculturable, pathogenic bacteria can maintain their infectivity (19, 27). These findings suggest that standard bacteriological methods are, indeed, inadequate to protect human health.

The fate of enteric bacteria in natural waters is affected primarily by factors such as light (4, 5, 13), temperature (3, 14, 44), other physical and chemical parameters (29, 41), and predation (3, 12, 25, 26). Sharp decreases in numbers of culturable enteric bacteria as a result of solar radiation have been reported (4, 5, 13). However, solar radiation does not produce bacterial lysis, and enteric bacteria remain morphologically intact after at least 5 days in an aquatic medium (0.2-μm filtered natural water) (4, 5). Most of those light-exposed cells were metabolically active, although they were nonculturable (4). Therefore, solar radiation does not reduce the total number of enteric bacteria present in aquatic ecosystems. Increases in temperature have been related to greater decreases of culturable enteric bacteria in aquatic ecosystems (1, 3, 9, 14). However, the effect of temperature on nonculturable enteric bacteria in natural water samples is unknown. Diverse physical and chemical factors, such as salinity and antibiotic substances produced by other bacteria and algae, have been reported to result in a decrease in the number of culturable enteric bacteria in natural aquatic media (0.2-μm-filtered natural water) (10, 27, 29, 31, 34, 41); but no decrease in the total number of enteric bacteria has been reported (19, 29). Therefore those physical and chemical factors do not contribute to the decrease in the total number of enteric bacteria in natural waters.

Protist predation has been shown to be a major factor responsible for the decay in the CFU of enteric bacteria in natural samples (1, 3, 12, 14, 25, 26, 34). However, these decreases in CFU could also be the result of either a loss of culturability or elimination by factors other than protistan grazing, such as attack by predatory bacteria (e.g., *Bdellovibrio* spp.) or bacteriophages (17). However, Garcia-Lara et al. (15) showed that bacteriophages have no significant effects on the survival of enteric bacteria in seawater.

Since protist predation implies ingestion and digestion of prey, this may be one of the factors responsible for the decay in the total number of enteric bacteria in aquatic ecosystems. González et al. (17) showed by using fluorescently labeled bacteria (FLB) that protists, both flagellates and ciliates, ingested and digested enteric bacteria in both freshwater and marine samples. They also demonstrated that the decrease in the total number of FLB throughout their experiments was due only to protist predation (17). Size-selective grazing by protists has also been reported (2, 8, 18, 28, 44) and may lead to significant differences in total counts of a bacterial species over time if target bacterial preys of different sizes are tested. Nevertheless, FLB cannot be cultured (that is, their

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CFU cannot be calculated), because they are heat-inactivated cells. Thus, FLB counts cannot be compared with CFU counts of unstained enteric bacteria.

In this work we used culturable FLB (rhodamine-stained bacteria [RSB]) to characterize the culturability of enteric bacteria in natural water samples. Moreover, by using enteric RSB, we were able not only to classify the enteric bacteria as culturable and nonculturable cells, but also to determine the total number of enteric bacteria removed from the ecosystem. The actual importance of protist predation for the removal of enteric bacteria in natural aquatic ecosystems was analyzed. A practical definition of bacterial death was also formulated.

MATERIALS AND METHODS

Samples. Sampling sites were located in the Butrón River, Spain, and La Salvaje beach, Spain. All samples were collected just beneath the surface of the water, and those from the marine ecosystem were collected 500 m from the coast. These ecosystems have been described previously (3, 16, 22).

Microorganisms. Bacterial strains used in this study were *Escherichia coli* ATCC 11775 and *Enterococcus faecalis* ATCC 19433.

Preparation of inocula. (i) Preparation of RSB. RSB were prepared basically by the procedure of Landry et al. (23). Some modifications were used to obtain the best results both in culturability and in staining quality. This resulted in differences in the preparation of RSB from *E. coli* and *E. faecalis* cells. Bacterial cultures were grown in nutrient broth at 28°C. *E. coli* cells were harvested by centrifugation (3,000 × *g* for 20 min) at exponential phase, and *E. faecalis* cells were harvested at stationary phase. Pellets were washed three times with phosphate-buffered saline (PBS) and suspended in a solution of dithioerythritol (40 mg ml^{-1}; Sigma Chemical Co., St. Louis, Mo.) in 0.2-μm-filtered seawater. After incubation at 4°C for 4 h (*E. coli*) or 3 h (*E. faecalis*), the dithioerythritol solution was removed by centrifugation and the cells were suspended in 10 ml of staining solution. The staining solution must be prepared just before use and consisted of 10 mg of rhodamine isothiocyanate (Sigma Chemical Co.), 10 ml of carbonate-bicarbonate buffer (pH 9), and 0.4 ml of acetone. Sonication helps to dissolve the stain. *E. coli* cells were incubated in the staining solution for 4 h at 12°C in the dark with shaking (100 rpm). *E. faecalis* cells were incubated for 1 h in the staining solution diluted 1:10 with the carbonate-bicarbonate buffer. Cells were washed three times with PBS and used immediately after preparation.

(ii) Preparation of NSB. Nonstained bacteria (NSB), both *E. coli* and *E. faecalis*, were prepared at the same time as the RSB. The NSB were processed as for RSB except that they were incubated in 10 ml of the carbonate-bicarbonate buffer instead of in the staining solution.

(iii) Preparation of FLB. FLB were prepared as described by Sherr et al. (38). *E. coli* and *E. faecalis* cells were harvested as above and washed three times in a 0.05 M Na_2HPO_4–0.85% NaCl (pH 9) solution. Pellets were suspended in 10 ml of the phosphate solution, and 2 mg of 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (Sigma Chemical Co.) was added. The suspensions were incubated in a water bath at 60°C for 2 h. The incubation was followed by three washes and suspension in PBS. Aliquots (2 ml) were frozen at 20°C in 5-ml plastic vials. Before use, FLB were thawed and slightly sonicated (four 1-s bursts at the 30-W power level) to disperse any bacterial clumps.

Experimental design. Natural water samples (200 ml) were distributed into several 500-ml glass flasks. Samples with and without natural microbiota were prepared in different ways. Filtered samples were prepared by passing natural waters through 0.2-μm cellulose filters (Millipore Corp., Bedford, Mass.). Untreated samples were directly distributed into corresponding flasks. RSB, NSB, and FLB were inoculated to a final density of 10^7 cells ml^{-1}. Inoculum size was determined by direct counts (see below). Each inoculum type was added to samples both with and without natural microbiota. Each sample was inoculated with only one inoculum type. Incubations were carried out in the dark with shaking (150 rpm) at in situ temperature for 5 days. Aliquots were collected daily, and those taken for direct counts were preserved with tetraborate-buffered Formalin (2% final concentration).

Statistical comparisons for each bacterial species were performed by using three-way analysis of variance with replication ($n = 4$) and unplanned comparisons, by the method of Sokal and Rohlf (42).

Enumeration of inoculated cells. For samples inoculated with NSB, CFU numbers were determined both in the absence and in the presence of natural microbiota. However, direct counts of NSB were determined only in the absence of natural microbiota. For samples inoculated with RSB, CFU and direct counts were performed both in the presence and in the absence of natural microbiota. For samples inoculated with FLB, only direct counts could be carried out since FLB are heat-treated cells (38).

(i) CFU counts. CFU counts of *E. coli* NSB and RSB were done on a selective medium, Levine eosin-methylene blue agar, at 35°C for 24 h. CFU counts of *E. faecalis* NSB and RSB were performed on a selective medium specific for this bacterial species, M-*Enterococcus* agar, at 35°C for 48 h. *E. coli* and *E. faecalis* CFU counts on selective and nonselective media (Trypticase soy agar supplemented with 0.3% glucose and 0.5% yeast extract) were compared over time in 0.2-μm-filtered water samples. No significant differences were obtained (data not shown).

(ii) Direct counts. Total numbers of NSB were determined by the acridine orange direct count method (20). Total numbers of RSB and FLB were counted on unstained 0.2-μm polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.) by epifluorescence microscopy at a magnification of ×1,250. Cell volumes (V) were calculated by the equation $V = [(4/3)\pi(W/2)^3] + [\pi(W/2)^3 \times (L - W)]$; bacterial length ($L$) and width ($W$) were measured from enlarged photographs projected on a screen. Volumes were compared by analysis of variance (42). Volumes and decay of RSB and FLB total numbers during our experiments in the presence of natural microbiota were correlated by using the Spearman coefficient of rank correlation (42).

Characterization of enteric bacteria. From RSB counts, *E. coli* and *E. faecalis* were characterized as culturable, nonculturable, and dead cells. Culturable cells were those estimated by CFU counts. Nonculturable cells included the fraction of cells undetected by CFU counts but enumerable by direct counts, that is, direct counts minus CFU counts. The number of dead cells was determined as the difference between the initial direct counts (at $t = 0$) and the direct counts at a specific time (0, 2, and 5 days).

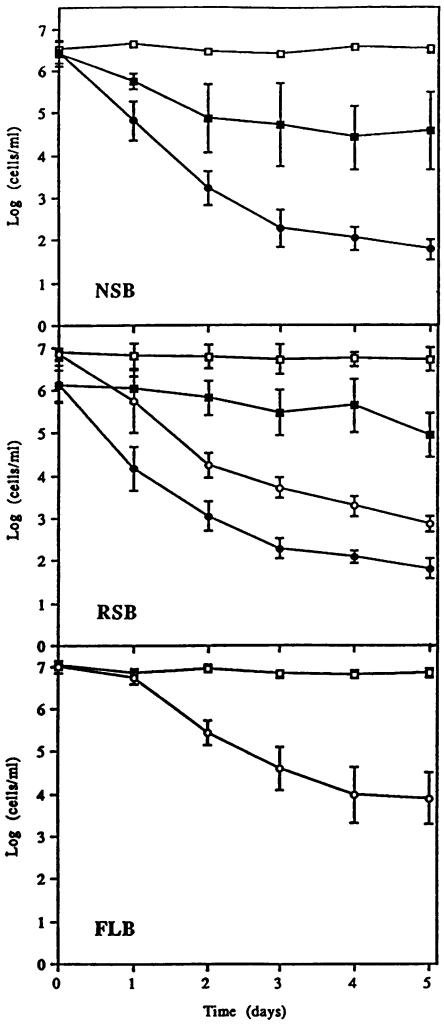

FIG. 1. Comparative counts of *E. coli* NSB, RSB, and FLB in a freshwater ecosystem. The figure shows CFU counts in the absence (■) and presence (●) of natural microbiota and direct counts in the absence (□) and presence (○) of natural microbiota. Bars indicate ± 1 standard deviation.

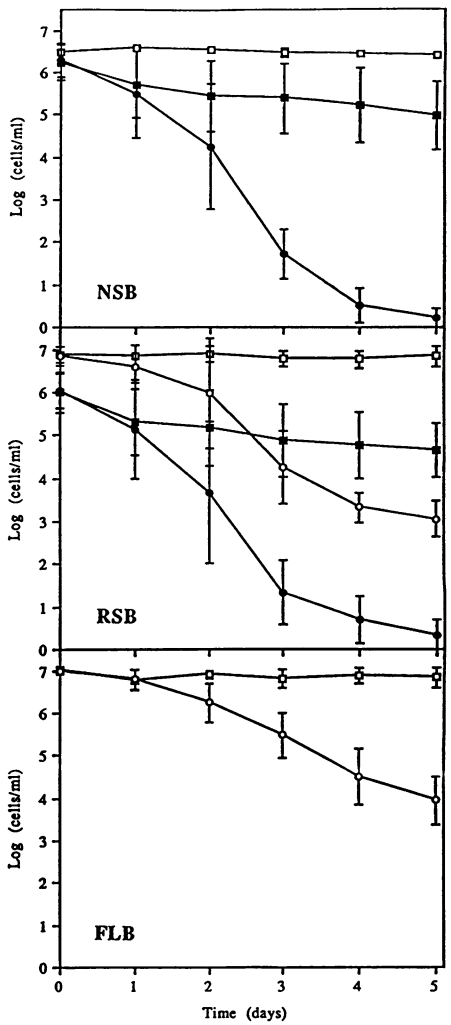

FIG. 2. Comparative counts of *E. coli* NSB, RSB, and FLB in a marine ecosystem. Symbols as in Fig. 1.

RESULTS

CFU counts. In samples without natural microbiota, there were no significant differences of CFU counts between NSB and RSB. This was true for both *E. coli* (Fig. 1 and 2) and *E. faecalis* (Fig. 3 and 4) in both freshwater and seawater samples.

In samples with natural microbiota, CFU numbers showed significant decreases ($P < 0.001$) with time. Similar decreases between CFU counts of NSB and RSB were observed (Fig. 1 to 4). Comparisons of CFU counts at the end of our experiments showed greater ($P < 0.001$) decreases in samples with natural microbiota than in samples without natural microbiota.

Direct counts. In samples without natural microbiota, the total number of NSB, RSB, and FLB remained constant throughout the incubations (Fig. 1 to 4). However, in freshwater and marine samples with natural microbiota, RSB and FLB direct counts decreased significantly with time for both *E. coli* ($P < 0.001$) and *E. faecalis* ($P < 0.01$) (Fig. 1 to 4). Comparison of samples with and without natural microbiota showed that the decrease of RSB and FLB in the presence of natural microorganisms was greater ($P < 0.001$) than in their absence in all cases (Fig. 1 to 4).

In samples with natural microbiota, significant differences ($P < 0.05$) between direct counts of *E. coli* RSB and *E. coli* FLB were observed during the incubation period. However, no significant differences between direct counts of *E. faecalis* RSB and *E. faecalis* FLB were obtained in our experiments carried out in the presence of natural microbiota (Fig. 1 to 4).

Significant differences ($P < 0.001$) were observed between volumes of NSB, RSB, and FLB. RSB and NSB had similar volumes for *E. coli* (2.10 ± 0.75 μm^3) and *E. faecalis* (0.47 ± 0.12 μm^3). Nevertheless, RSB volumes were significantly greater than FLB volumes for *E. coli* (0.83 ± 0.25 μm^3) and *E. faecalis* (0.34 ± 0.11 μm^3). Thus, the ratio of the RSB volume to the FLB volume for *E. coli* was 2.53 and that for *E. faecalis* was 1.38. The volumes of RSB and FLB (for *E. coli* and *E. faecalis*) were correlated with the decreases in total numbers of RSB and FLB (difference between initial and final total counts) during our experiments (Fig. 1 to 4). Regression analysis (42) showed significant relationships ($P < 0.01$) between volumes and decreases of total counts

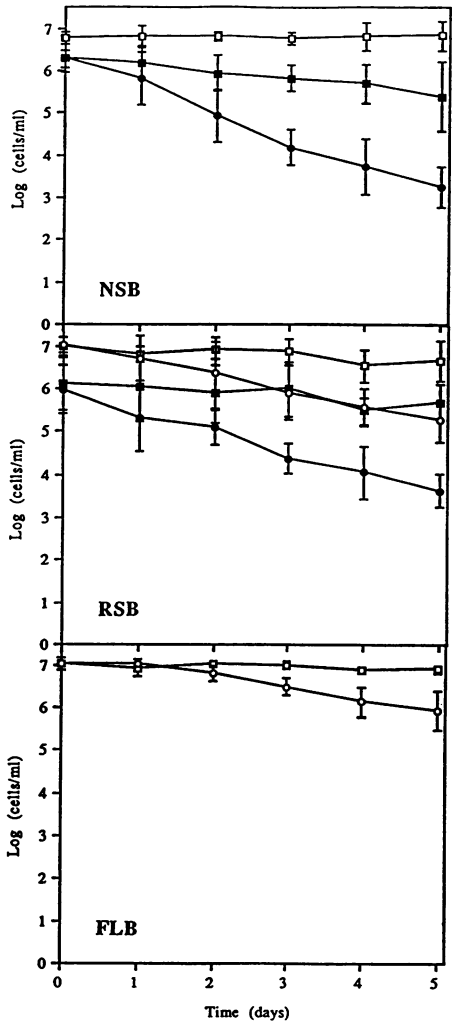

FIG. 3. Comparative counts of *E. faecalis* NSB, RSB, and FLB in a freshwater ecosystem. Symbols as in Fig. 1.

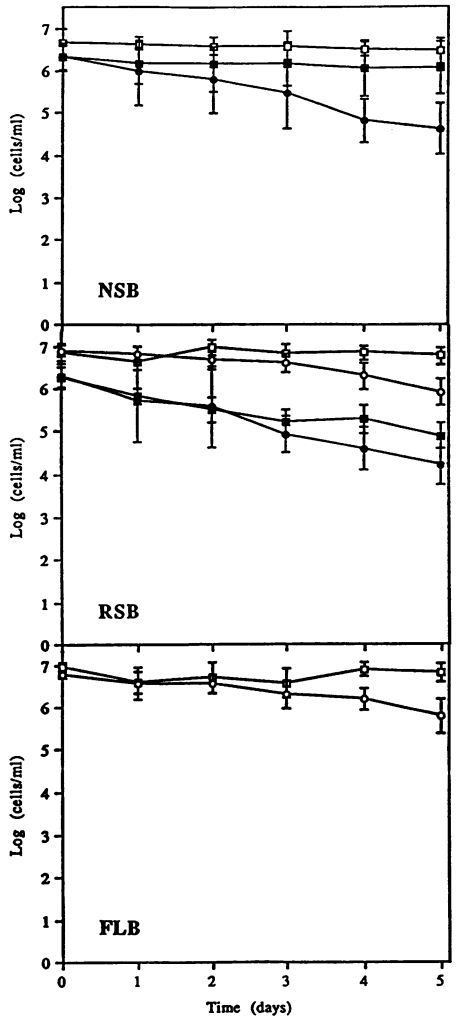

FIG. 4. Comparative counts of *E. faecalis* NSB, RSB, and FLB in a marine ecosystem. Symbols as in Fig. 1.

during the experiments, explaining more than 99% of the differences in the decrease in total numbers between RSB and FLB.

Comparisons between CFU and direct counts. CFU and direct counts for RSB were compared both in the absence and in the presence of natural microbial populations. Significant differences were observed both in the absence ($P < 0.005$ for *E. coli*, and $P < 0.05$ for *E. faecalis*) and in the presence ($P < 0.001$ for *E. coli*, and $P < 0.05$ for *E. faecalis*) of natural microbiota. By the end of the incubations carried out in subsamples with natural microbiota, total numbers were approximately 1 (*E. faecalis* in seawater samples) to 4 (*E. coli* in seawater samples) orders of magnitude greater than CFU counts, depending on the strain of enteric bacterial species and the water sample (Fig. 5).

Figure 5 shows the characterization of culturable, nonculturable, and dead cells of *E. coli* and *E. faecalis* throughout our experiments carried out by using samples with and without natural microbial populations. In the presence of natural microbiota, an increase in the number of dead cells was observed for *E. coli* and *E. faecalis* in both freshwater and seawater samples. In the absence of natural microbiota only culturable and nonculturable cells were characterized because no decrease of direct counts was detected. Nonculturable cells were an important fraction of total number of cells in samples with and without natural microbiota.

DISCUSSION

The decrease in numbers of culturable enteric bacteria in natural waters in the presence of natural microbiota has been used to show that protist predation is the major factor responsible for the elimination of enteric bacteria from natural aquatic ecosystems (1, 3, 12, 24, 25, 27, 34). However, the problem with this methodology is that decreases in numbers of enteric bacteria correspond only to the decreases in CFU and do not provide any information about the total number of enteric bacteria (culturable and nonculturable). Thus, it is not possible to determine the real effect of protists on the survival of an enteric bacterial species. By using RSB, which can be cultured and enumerated by fluorescence microscopy, one is able to estimate total numbers of a specific enteric bacterial species and hence evaluate the total effect of predators on the survival of enteric bacteria.

However, before using this approach with RSB it is necessary to check whether counts of NSB, RSB, and/or

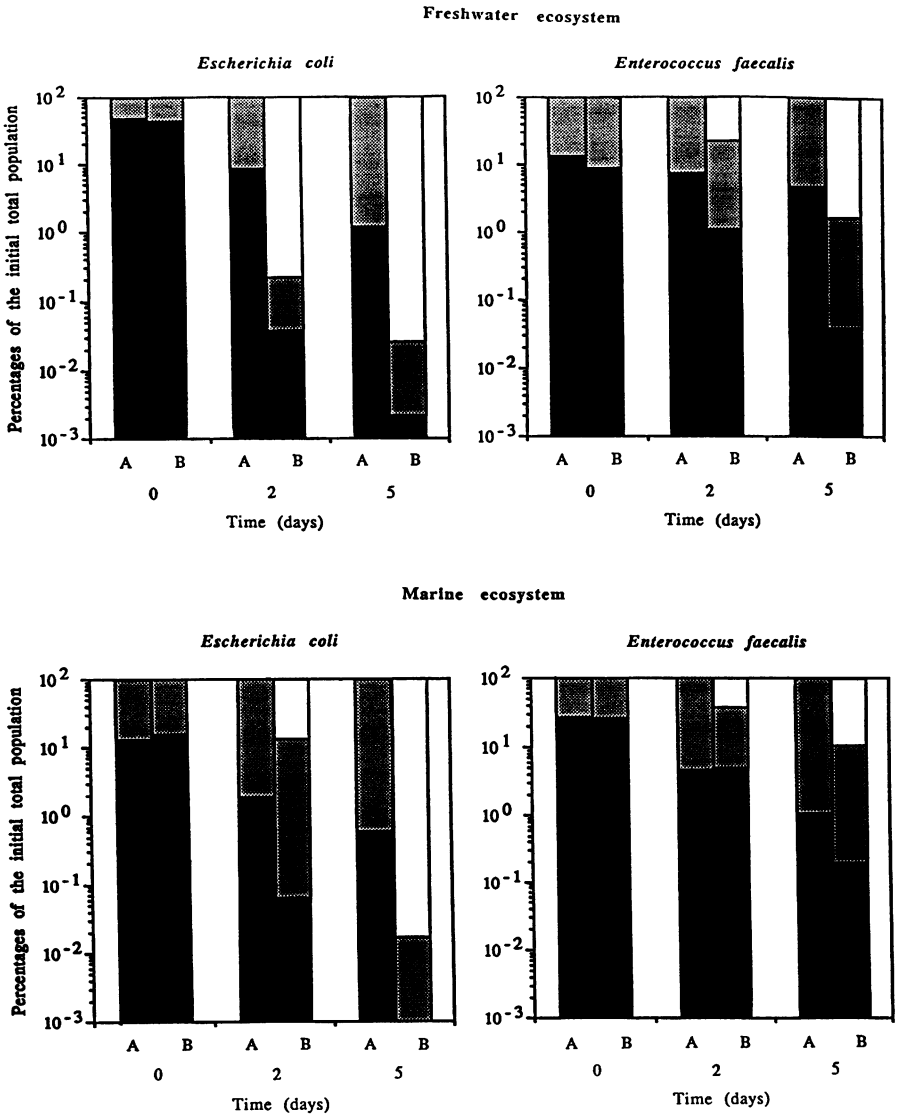

FIG. 5. Characterization of culturable (■), nonculturable (▨), and dead (□) cells of *E. coli* and *E. faecalis* in freshwater and marine ecosystems in the absence (A) and presence (B) of natural microbiota.

FLB are comparable. Since no differences were observed between CFU counts of NSB and RSB (Fig. 1 to 4), data obtained from RSB enumerations can be compared with those for NSB. Our results agree with those of other authors (1, 3, 12, 24–26, 34), who reported greater decreases in CFU of enteric bacteria in the presence than in the absence of natural microbiota. In the absence of natural microbiota, direct counts of RSB were also comparable with those of NSB since both remained constant throughout the experiments (Fig. 1 to 4). This also agrees with the published reports of other workers (19, 29, 36, 45), who showed constant total counts of enteric bacteria in the absence of natural microbiota during their experiments. Moreover, these results lead to the conclusion that neither loss of fluorescence nor spontaneous lysis of RSB occurred during our 5-day experiments. Thus, RSB enumeration was a reliable indicator of both total numbers and CFU of enteric bacteria throughout our experiments and could be used to (i) characterize the culturability of enteric bacteria in unscreened natural water samples and (ii) determine the effect of microbial predators on these organisms.

As a consequence of recent studies (4, 5, 19, 29, 36, 45) and this study, the classic concept of bacterial death as stated by Postgate (32), i.e., a loss in the ability to form colonies on a suitable culture medium, must be redefined. We now understand a dead bacterium to be one which has lost its morphological integrity (36, 37), and so the only way to detect dead bacteria is by decreases of direct counts over time (Fig. 5). Thus bacterial death would be a synonym for cellular lysis, and bacterial survival should be applied to this definition. Injured bacteria would be cells which cannot be cultured on standard bacteriological media (nonculturable cells) but which remain morphologically intact (4, 5, 19, 29, 36). They can still be active cells (4, 19, 35, 36), and if they are pathogenic bacteria they can be infective (19, 27).

From the results reported here, it is possible to compare and characterize the progressive dormancy and death of the experimental enteric bacteria when placed in natural water

with and without natural microbial assemblages (Fig. 5). In the absence of natural microbiota, enteric bacteria can be classified only as culturable cells and nonculturable cells since no decrease in direct counts was detected. In the presence of natural microbiota, enteric bacteria can be classified as culturable cells, nonculturable cells (or somnicells), and dead cells. The culturable-cell fraction, estimated by CFU counts, is the only fraction usually reported in survival studies in the presence of natural microbiota. Direct counts of RSB allow us to estimate the total number of living enteric bacteria as the sum of culturable and nonculturable cells, that is, the viviform population (36). Dead cells are lysed bacteria, so they cannot be enumerated by direct counts. As a consequence of the above results, we can affirm that CFU counts really underestimate the total number of viviform enteric bacteria in aquatic ecosystems and are not an appropriate methodology for the evaluation of water quality.

Predation is the only known natural factor which eliminates enteric bacteria from the aquatic ecosystems, since a decrease in the total number of enteric bacteria was not observed either in the dark and the absence of natural microbiota (19, 29, 36, 45; see also this study) or in the light (4, 5). In natural aquatic ecosystems, predation can be exerted by protozoa or by other agents such as predatory bacteria (e.g., *Bdellovibrio* spp.) and bacteriophages (6, 17, 30, 33, 37, 39). Since predatory bacteria and bacteriophages appear to lyse only active bacteria (7, 16, 21, 40) and FLB are heat-inactivated cells, decreases in FLB numbers in the presence of natural microbiota could only be the result of protist predation (see reference 17 for more details). Thus, the differences between RSB and FLB direct counts might allow us to estimate the possible effect of predatory agents other than protists on the survival of enteric bacteria. For *E. faecalis*, no significant differences were observed between RSB and FLB direct counts, which means that there was no significant effect of predatory bacteria and bacteriophages on *E. faecalis* survival. For *E. coli* these differences were small and may also be explained by other causes. For instance, size-selective grazing has been reported by several authors (2, 8, 18, 28, 43). In our experiments, we observed that the differential decrease in the numbers of RSB and FLB enteric bacteria can be explained by a positive relationship with the size of the inoculated bacteria (FLB and RSB). Moreover, Landry et al. (23) reported that a marine phagotrophic nanoflagellate (*Paraphysomonas vestita*) could discriminate against FLB in favor of RSB. Consequently, the possible effect of predatory bacteria and bacteriophages on the survival of enteric bacteria in the current set of experiments was insignificant. Protists were the major factor responsible for the decay in the total number of enteric bacteria. This is in agreement with the results of Garcia-Lara et al. (15), who reported no significant effects of bacteriophages on the survival of fecal bacteria in seawater.

In conclusion, enteric bacteria are present in natural aquatic ecosystems as culturable and nonculturable cells. Nonculturable enteric bacteria are undetectable by standard bacteriological methods. Bacterial death and survival should be assessed in terms of decreases in the total number of enteric bacteria, i.e., in terms of their viviform population (36), and not to decreases in CFU. Moreover, protozoan predation is the main cause of death of enteric bacteria in aquatic ecosystems, and the effects of predatory bacteria and bacteriophages on enteric bacteria survival are not significant both in marine and in freshwater ecosystems.

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