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Mortality of Fecal Bacteria in Seawater

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We propose a method for determining the mortality rate for allochthonous bacteria released in aquatic environments without interference due to the loss of culturability in specific culture media. This method consists of following the disappearance of radioactivity from the trichloroacetic acid-insoluble fraction in water samples to which [^{3}H]thymidine-prelabeled allochthonous bacteria have been added. In coastal seawater, we found that the actual rate of disappearance of fecal bacteria was 1 order of magnitude lower than the rate of loss of culturability on specific media. Minor adaptation of the procedure may facilitate assessment of the effect of protozoan grazing and bacteriophage lysis on the overall bacterial mortality rate.

Work initiated more than 100 years ago has demonstrated the rapid disappearance (as determined by agar counts) of fecal bacteria released into natural aquatic environments (1, 5, 7). Several papers have dealt with the effect of environmental factors, including salinity, temperature, light, and the presence of predators, on the rate of disappearance (1, 3, 10–12).

Recently, however, suspicions have been aroused about the validity of the classical enumeration techniques in which agar plates and selective media are used. For instance, Colwell and co-workers (4, 6, 14, 17, 21) have shown very clearly that when enteric bacteria are introduced into seawater, they maintain cellular integrity for several days to several weeks but rapidly lose their ability to be cultivated by classical methods. When the nonculturable cells are examined for metabolic activity, they are viable, and in some cases they have been found to have retained their virulence (4, 6). It follows from these findings that the rate of loss of culturability on classic agar media, which constitutes the basis of all previous work on the disappearance of fecal bacteria in aquatic environments, strongly underestimates the risk of survival of these bacteria.

Therefore, reassessment of the models currently used for predicting the bacteriological quality of aquatic systems subject to fecal contamination is urgently needed. Actual rates of disappearance of enteric bacteria released into natural waters must be redetermined, and the processes responsible for the disappearance should be identified. Considerable progress has been made recently in our knowledge of the factors responsible for the mortality of autochthonous bacteria in aquatic ecosystems. Grazing by nanoflagellates has been identified as a major process (9, 13). On the other hand, bacteriophages have been found to be very abundant in aquatic environments, and it has been suggested that these organisms play a significant role in bacterial mortality (2, 16), although direct measurements of rates of virus-induced bacterial mortality have not been made. At present how these concepts apply to the mortality of allochthonous bacteria (like fecal bacteria) is completely unknown.

In this paper we propose a procedure for determining the actual rate of disappearance of enteric bacteria released into natural waters without interference due to loss of culturability. Our procedure also may facilitate quantification of the roles that different factors, including protozoan grazing and viruses, play in bacterial mortality. The procedure described below is adapted from the method proposed by Servais et al. (18, 19) for measuring the rate of mortality of autochthonous aquatic bacteria in natural waters. This method consists of prelabeling the DNA of a natural assemblage of bacteria by using [^{3}H]thymidine and following the rate of disappearance of radioactivity from trichloroacetic acid (TCA)-insoluble material. As DNA is basically a conservative molecule, undergoing little turnover within the cell (if we exclude the processes of DNA repair, which are probably quantitatively insignificant), the decrease in radioactivity from the DNA constitutes a measure of bacterial mortality.

In a first experiment, we followed the rate of disappearance in seawater (collected off Ostend, Belgium) of three [^{3}H]thymidine-prelabeled strains of fecal bacteria, an *Escherichia coli* strain (recently isolated from human feces), *Salmonella typhimurium* ATCC 14028, and *Streptococcus faecium* ATCC 10541. These strains were grown for 4 to 6 h in Luria-Bertoni medium (10 g of tryptone [Difco Laboratories] per liter, 5 g of yeast extract [Difco] per liter, 10 g of NaCl per liter) in the presence of 100 nM [*methyl*-^{3}H]thymidine (30 to 40 Ci/mmol; Amersham) (8), centrifuged at 5,000 rpm for 15 min, rinsed three times by resuspension in sterile seawater and recentrifugation, and finally added to 1 liter of seawater at a final concentration of about 0.05×10^9 cells per liter (about 5% of the total bacterial population). The seawater was incubated in the dark at 11°C (in situ temperature), and samples were taken twice a day for 4 days to determine the cold 5% TCA-insoluble radioactivity and the number of CFUs on specific agar media. For the latter determination, we used the spread plate method with salmonella-shigella agar and KF agar (ADSA-Micro) for enumerating *Salmonella typhimurium* and *Streptococcus faecium*, respectively, using 24- and 48-h incubations at 30°C. To determine the number of *E. coli* CFUs, we used the classical membrane technique that is routinely used in sanitary control of seawater bathing areas; membranes (pore size, 0.45 μm) were incubated at 44°C for 24 h on Tergitol Agar (Merck) containing 1% TTC. The experiments with a freshly collected untreated seawater sample and an autoclaved sample were run in parallel. In the latter case, we also determined plate counts of fecal bacteria on a nonspecific medium (tryptocasein agar incubated at 30°C for 24 to 48 h) and microscopic

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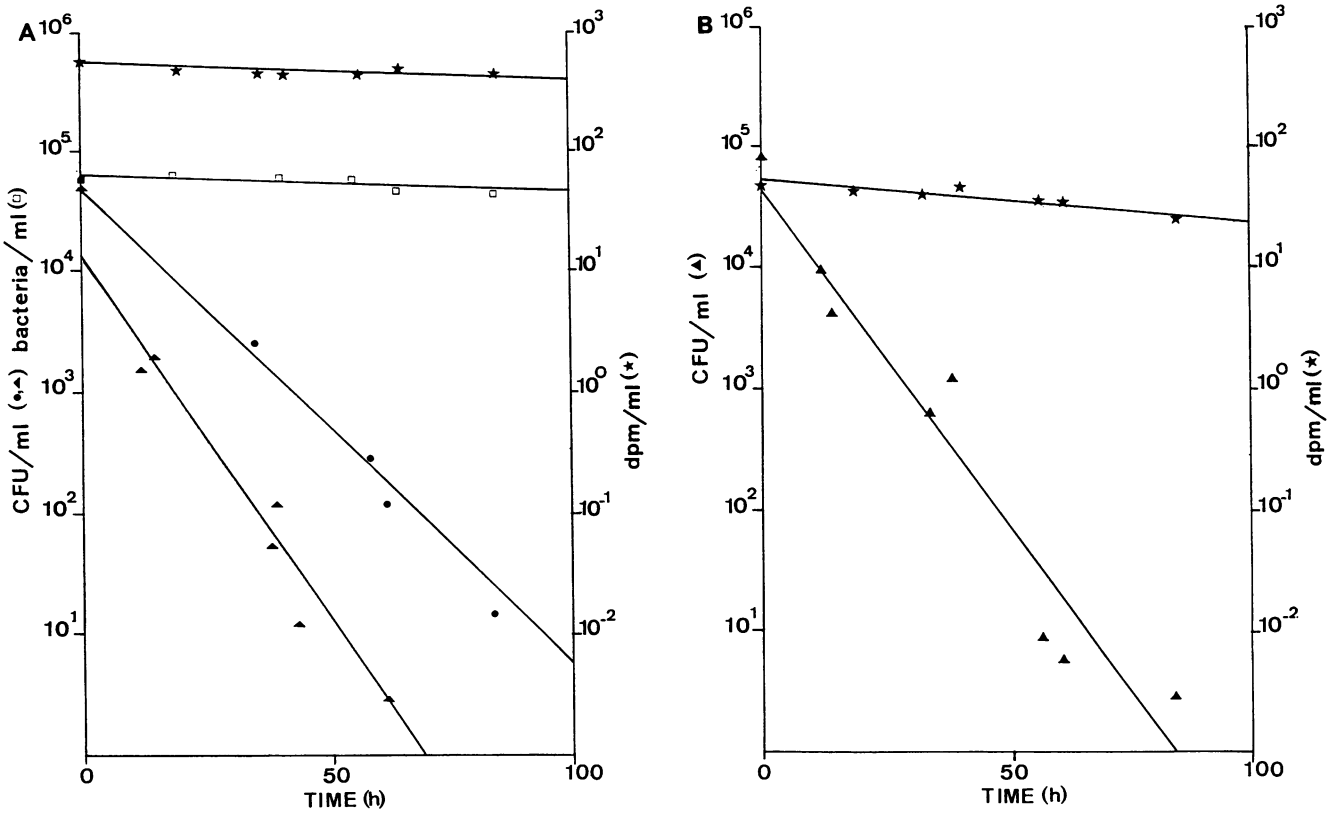

FIG. 1. Loss of culturability of *E. coli* on specific medium (▲) and nonspecific medium (●), decrease in direct microscopic counts (□), and decrease in radioactivity in TCA-insoluble material (★) in filtered (pore size, 0.2 μm) and autoclaved seawater (A) and in untreated seawater (B). The seawater sample was obtained from the Belgium coastal zone at Ostend in April 1990.

direct counts after DAPI staining (15). The results obtained with *E. coli* are shown in Fig. 1. In our experiments all of the rates of decrease were determined by a linear regression analysis of the log of either radioactivity or bacterial numbers as a function of time. In sterile seawater, slow and similar rates of decrease were observed for the radioactivity associated with TCA-insoluble material (0.0008 h^{-1}) and for the direct counts (0.001 h^{-1}), while much more rapid losses of culturability occurred in specific agar medium (0.140 h^{-1}) and in nonspecific agar medium (0.090 h^{-1}). Similarly, in untreated seawater the loss of culturability on specific medium was 1 order of magnitude greater than the rate of mortality deduced from the rate of disappearance of TCA-insoluble radioactivity, which was, however, three times higher than the rate of mortality in the sterile control.

Quite similar results were obtained with the other two strains (Table 1), confirming the conclusion of previous authors that nonculturable but morphologically and genetically intact fecal bacteria can exist in natural waters. The overestimation of mortality rates by agar plating methods was less pronounced for the *Streptococcus faecium* strain than for the other two strains. In any case, however, our

TABLE 1. Rates of disappearance of three fecal bacteria in seawater[a]

Organism	Sample	Rate of disappearance (10^{-3} h^{-1}) as determined by:			
		Plate count method		Direct microscopic observation	^{3}H-label method
		Specific medium	Nonspecific medium		
E. coli	Untreated seawater	128			7.6
	Filtered (pore size, 0.2 μm) and autoclaved seawater	140	90	1	0.8
Salmonella typhimurium	Untreated seawater	160			6.0
	Filtered (pore size, 0.2 μm) and autoclaved seawater	121	70	0.38	0.23
Streptococcus faecium	Untreated seawater	43			9.90
	Filtered (pore size, 0.2 μm) and autoclaved seawater	19	14	0.80	1

[a] The rates of disappearance at 11°C were estimated from plate counts on specific medium and nonspecific medium, from direct microscopic observations, and from the decreases in radioactivity in macromolecular material (see text). The seawater was collected in the Belgium coastal zone at Ostend in April 1990.

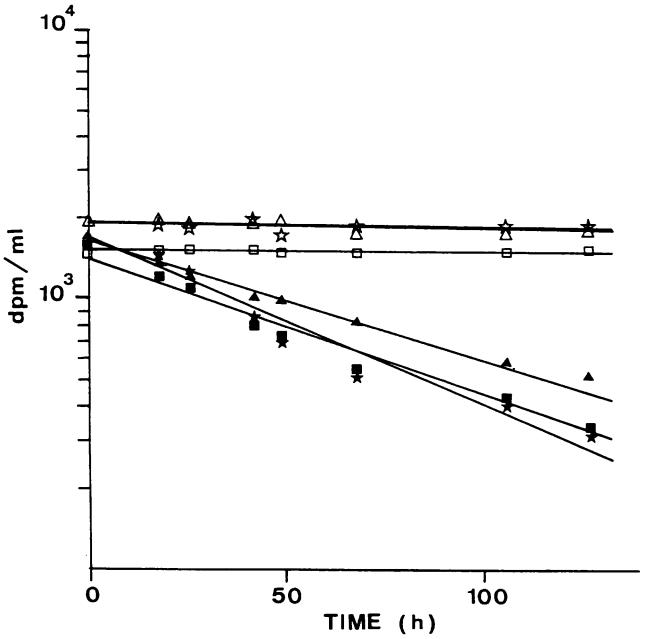

FIG. 2. Decrease in radioactivity in TCA-insoluble material after addition of labeled *E. coli* (☆ and ★), *Salmonella typhimurium* (□ and ■), and *Streptococcus faecium* (△ and ▲) to untreated seawater (solid symbols) and to seawater that was filtered (pore size, 0.2 μm) and contained inhibitors (open symbols).

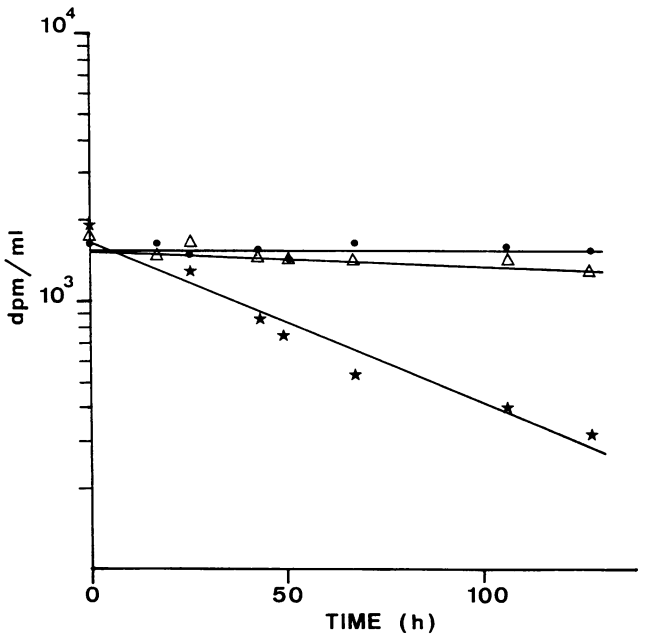

FIG. 3. Decrease in radioactivity in the material retained on a 0.2-μm-pore-size filter after addition of labeled *E. coli* to filtered (pore size, 0.2 μm) and autoclaved seawater (●), to nonautoclaved filtered (pore size, 0.2 μm) seawater (△), and to untreated seawater (★).

method in which [^{3}H]thymidine-labeled bacteria were used appeared to be more appropriate for studying the fate of allochthonous bacteria in natural waters.

With only minor modifications, our method may also be useful for assessing the roles of various processes in the overall mortality rate. In order to determine the role of protozoan grazing in the disappearance of fecal bacteria in seawater, we compared the rates of decrease of TCA-insoluble radioactivity in a sample of untreated seawater and in a sample of seawater filtered through a 2-μm-pore-size membrane filter (Nuclepore Corp.) to which a mixture of cycloheximide and colchicine (final concentrations, 200 and 100 mg/liter, respectively) had been added. The latter treatment was intended to retain most protozoa and to inhibit the activity of the protozoa that passed through the 2-μm-pore-size filter (20). The contribution of protozoan grazing to mortality could be calculated as the difference between the rates of decrease in radioactivity in the two samples. The results of this experiment performed with *E. coli*, *Salmonella typhimurium*, and *Streptococcus faecium* are shown in Fig. 2. In all three cases, the mortality rates for the samples that were filtered and contained inhibitors were very low, while the controls exhibited important decreases, suggesting that protozoan grazing was the most important factor responsible for bacterial mortality in the seawater samples. In Fig. 2, it appears that the mortality rates of all three strains were quite similar, suggesting that the morphological and biochemical differences of these organisms did not result in any specificity in protozoan grazing in this case.

In the assay described above, mortality due to bacteriophage attack would not be detected. Indeed, during virus-induced lysis, most of the ^{3}H-labeled DNA would be converted into phage material, which would remain in the TCA-insoluble fraction (18). In order to assess the possible role of bacteriophages in bacterial mortality, we compared the rate of disappearance of radioactivity retained on 0.2-μm-pore-size membranes without TCA precipitation after addition of prelabeled *E. coli* to (i) untreated seawater (sample A), (ii) seawater filtered through 0.2-μm-pore-size membranes (sample B), and (iii) autoclaved seawater filtered through 0.2-μm-pore-size membranes (sample C) (Fig. 3). The large difference observed in the rates of decrease in radioactivity between samples A and B confirms that particles retained by 0.2-μm-pore-size filtration (namely, protozoans and possible bacterial predators) play the major role in overall *E. coli* mortality. A small but significant difference was observed between samples B and C. This difference represents the role played in *E. coli* mortality by thermosensitive agents that are smaller than 0.2 μm in diameter, including bacteriophages and other possible toxins. Therefore, these results set an upper limit to the contribution to mortality due to bacteriophage lysis. In this experiment, this contribution was rather limited compared with the contribution of protozoan grazing (Fig. 3).

We concluded that our procedure offers a powerful tool for determining the fate of allochthonous bacteria released into aquatic environments and for studying the factors responsible for the mortality of these bacteria. Our method overcomes the problems of culturability on selective media for many bacterial strains.

Our method may be helpful for determining the role of specific processes, such as protozoan grazing and bacteriophage lysis, in the overall mortality of allochthonous bacteria. The range of application of this method is not restricted to the study of fecal bacteria, as the procedure could also be used for assessing the survival of genetically engineered microbes released into aquatic environments.

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