APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1995, p. 1888–1896 Vol. 61, No. 5
0099-2240/95/$04.00+0

Survival of Fecal Microorganisms in Marine and Freshwater Sediments

CHERYL M. DAVIES,* JULIAN A. H. LONG, MARGARET DONALD, AND NICHOLAS J. ASHBOLT

Australian Water Technologies, EnSight, West Ryde, New South Wales 2114, Australia

Received 21 November 1994/Accepted 1 March 1995

The survival of culturable fecal coliforms, fecal streptococci, and *Clostridium perfringens* spores in freshwater and marine sediments from sites near sewage outfalls was studied. In laboratory studies, the inhibition of protozoan predators with cycloheximide allowed the fecal coliforms to grow in the sediment whereas the presence of predators resulted in a net die-off. *C. perfringens* spores did not appear either to be affected by predators or to die off throughout the duration of the experiments (28 days). Studies using in situ membrane diffusion chambers showed that, with the exception of *C. perfringens*, die-off of the test organisms to 10% of their initial numbers occurred in both marine and freshwater sediments within 85 days. The usual exponential decay model could not be applied to the sediment survival data, with the exception of the data for fecal streptococci. It was concluded that application of the usual decay model to the fecal coliform data was confounded by the complex relationship between growth and predation. The survival of seeded *Escherichia coli* in marine sediment was studied by using an enumeration method which detected viable but nonculturable bacteria. Throughout the duration of the experiment (68 days), the same proportion of *E. coli* organisms remained culturable, suggesting that sediment provides a favorable, nonstarvation environment for the bacteria.

A study commenced in 1989 to monitor microbial indicators and pathogens in marine waters in the vicinity of Sydney's three deepwater (80 m) ocean outfalls at North Head, Bondi, and Malabar. Surface and subsurface waters around the three outfalls, as well as inshore sediments off Malabar, were monitored. The study is ongoing, and results to date have been reported by Ashbolt et al. and Grohmann et al. (2, 12). Although the commissioning of the outfalls has resulted in reductions in beach water fecal microorganisms to acceptable levels, bacterial concentrations in inshore Malabar sediments have not decreased and enteric viruses persist in these sediments. These observations would suggest extended survival of microorganisms in sediments and are consistent with observations made previously by other workers (10). Alternatively, there may be additional unidentified sources of fecal pollution, such as urban runoff or onshore sediment transport from the deepwater outfalls.

Accumulation of indicator bacteria and viruses in sediments is well documented (25) and has been attributed to the sorption of the microorganisms to particles suspended in water, which then sediment out. Sediments may contain 100 to 1,000 times as many fecal indicator bacteria as the overlying water (2, 30). Therefore, the period of pathogen survival and associated particle transport into water used for recreation and fisheries is of concern to regulatory authorities.

Several factors have been proposed which considerably reduce the survival rates of fecal bacteria in the aquatic environment. Sunlight is thought to be the single most important factor contributing to the death of these bacteria in seawater (5). Other factors include high salinity (24), the presence of toxic agents (16), predation and parasitism (8), and low nutrient concentrations (9). However, fecal bacteria have been shown to survive and, to a certain extent, even to grow in sediments (10, 14, 18). Bacteria adsorbed to sediment particles may be protected from the influence of such factors as UV radiation (4), high salinity (11), heavy metal toxicity (16), and attack by bacteriophage (26). Additionally, fecal bacteria are able to obtain nutrients associated with the sediment particles. The observed persistence of bacteria at fairly constant levels in sediments may be the result of a balance between the rate of their growth versus predation (19).

Methods traditionally used for enumerating bacteria in waters are generally unsuitable for sediments. Attached bacteria are not adequately dispersed by the usual practice of hand shaking, resulting in lowered counts (15). Consequently, much effort has been put into the separation of bacteria from sediment particles by various means. Generally, three main methods have been used: chemical dispersion, sonication, or homogenization. Sonication has been shown to be by far the most effective means for the separation of bacteria from particles (17, 20, 31), though it is often used in conjunction with a chemical desorbing agent.

In addition to inadequate dispersion, numbers of fecal bacteria may be underestimated because of the formation of viable but nonculturable (VNC) forms (27, 33). We are unaware of any previous studies on the adoption of the VNC state by bacteria in sediments. Also, survival studies have previously focused mainly on fecal coliforms in either marine or freshwater sediments. Therefore, this study focused on the survival of key fecal indicator bacteria, and the formation of VNC *Escherichia coli*, in aquatic sediments associated with coastal and inland sewage outfalls.

MATERIALS AND METHODS

Collection of sediments. For predation experiments, sediment was collected from the bottom of a pool just below the discharge point of a storm water drain (SWD) at Whale Beach, one of Sydney's northern beaches, and from a marine site adjacent to the Malabar deepwater outfall outlet (M5) (33°58.51′S, 151°17.88′E). For marine in situ studies, sediment was collected from a site 0.4 km offshore from the previous cliff edge outfall at Malabar (M2) (33°58.27′S 151°16.53′E) (Fig. 1). Marine sediments were collected with a pipe dredge (length, 30 cm; diameter, 8 cm; fitted with fine mesh at the rear end). Freshwater sediments for in situ studies were collected with a grab sampler (Van-Veen Grab; Water Board, Sydney, Australia) from Prospect Creek at Milperra, a site im-

* Corresponding author. Present address: Centre for Advanced Analytical Chemistry, CSIRO Division of Coal and Energy Technology, Private Mail Bag 7, Menai, NSW 2234, Australia. Phone: 61 2 710 6853. Fax: 61 2 710 6837.

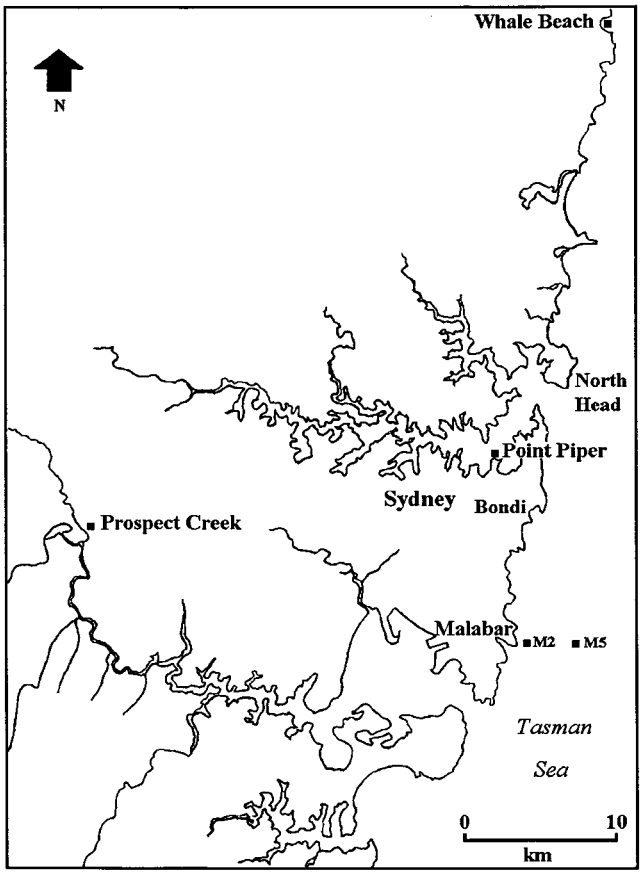

FIG. 1. Sampling and experiment sites.

pacted by overflow of raw sewage during storm events (Fig. 1). Sediments were stored on ice and transported back to the laboratory, where they were refrigerated until processing the next day.

Pretreatment of sediments in survival studies. All sediment samples withdrawn during predation in situ and VNC microcosms were mixed thoroughly, and 10 g was weighed out into 90 ml of sterile deionized water. These were sonicated at 100 W for 30 s (31) with a Braun Labsonic U ultrasonic probe (diameter, 19 mm) and allowed to settle for 10 min prior to withdrawal of the supernatant for enumeration of bacteria. Preliminary studies had shown that this treatment resulted in improved recovery of fecal bacteria from the sediments tested (data not shown).

Enumeration methods. Enumeration of bacteria was carried out by using Standards Association of Australia-approved procedures. The methods used were based on standard methods (1, 7), and any modifications made to improve recovery have been validated (3). Membrane filtration was used for the enumeration of fecal coliforms, fecal streptococci, and *Clostridium perfringens*. In each case, between 1 and 80 ml of the sediment supernatant (after settling for 10 min) was filtered through a 47-mm-diameter membrane filter (Millipore Corp., Bedford, Mass.) with a pore size of 0.45 μm. As bacteria do not significantly change their morphology in sediment (23), it was assumed that they would not form small, round, starved cells which are capable of passing through the membrane filter pores.

Fecal coliforms were enumerated by placing the membrane filter on top of a 15-ml layer of membrane fecal coliform agar (mFC; Difco Laboratories, Detroit, Mich.). Incubation was at 35°C for 4 h, followed by 14 h at 44.5°C, in metal containers in a water bath. All blue colonies greater than 1 mm in diameter on the membrane filter surface were counted and recorded as presumptive fecal coliforms. At least 10 colonies on membrane filters with 20 to 80 typical colonies were confirmed by inoculation into Lauryl Tryptose broth (Oxoid Ltd., Basingstoke, United Kingdom). After incubation overnight at 44.5°C, the production of gas and growth in tubes were recorded, and these colonies were confirmed as being fecal coliforms.

For the enumeration of fecal streptococci, the membrane filter was placed on a 15-ml layer of m-Enterococcus agar (Difco). Incubation was at 35°C for 48 h, in metal containers in a water bath. All light and dark red colonies on the surface of the membrane filters were counted and recorded as presumptive fecal streptococci. Confirmation of colony types followed transfer of membrane filters onto Bile Aesculin Azide agar (Oxoid) and incubation at 45°C for 1 h in a water bath. All colonies which produced a black color against the agar were confirmed as being fecal streptococci.

For the enumeration of *C. perfringens* spores, a portion of the sediment supernatant was heat shocked to 75 to 80°C for 10 min in a water bath and cooled before being filtered through a membrane filter. Enumeration of total *C. perfringens* (spores and vegetative cells) was carried out on non-heat-shocked sediment supernatant. For heat-shocked and non-heat-shocked samples, the membrane filters were placed on top of a 15 ml layer of Perfringens agar (Oxoid) (7) and incubated at 35°C, in an air-circulated incubator for 18 to 24 h, in an anaerobic jar with an anaerobic atmosphere generator (Oxoid). All black and grey colonies on the surface of the membrane filter were counted and recorded as presumptive *C. perfringens*. Confirmation was carried out by streaking colonies onto Tryptose Sulphite Cycloserine agar (E. Merck, Darmstadt, Germany) supplemented with 4-methylumbelliferyl phosphate, which was incubated at 35°C for 24 h in an anaerobic atmosphere. Colonies which fluoresced under long-wavelength UV light (366 nm) were confirmed as being *C. perfringens*. All numbers of bacteria were expressed as CFU per 100 g of wet sediment.

Predation studies. Closed-bottle microcosms were prepared by weighing out 150 g of sediment into a number of sterile conical flasks. Filter-sterilized (pore size, 0.2 μm) seawater (50 ml) was also placed into the flasks. To inhibit protozoan predation, 2 g of cycloheximide per 100 g of sediment (19) was added to half of the microcosms. Sediments were then stored loosely capped at 20°C in the dark without agitation. An initial sample was withdrawn from each, before incubation. Sampling was then carried out at regular intervals following removal of the overlying water with a sterile syringe with care being taken not to disturb the surface of the sediment. In the latter stages of the experiment it was necessary to take out more than 10 g of sediment so that the bacteria could be detected. Accordingly, several 10-g portions were weighed into several bottles each containing 90 ml of sterile deionized water, and the supernatants were pooled. The overlying water was replaced after sampling. The samples were pretreated by sonication and enumerated as described previously for fecal coliforms, fecal streptococci, and *C. perfringens spores.*

In situ microcosm survival studies. The membrane diffusion chambers consisted of three circular pieces of Perspex and were based on those described by Roper and Marshall (26). The two outer pieces were 6.5 mm thick, and the central piece was 25 mm thick. In the smaller chambers the lumen in the central spacer was 60 mm in diameter and accommodated up to 80 g of sediment. In the larger chambers the lumen in the central spacer was 80 mm in diameter and accommodated up to 180 g of sediment. A single sampling-filling port 15 mm in diameter was cut into the top of the central plate and fitted with a rubber stopper. The chamber components were cleaned with 70% ethanol to remove dust and other particles. Membranes were cut from sheets of polycarbonate (pore size, 0.2 μm) (Nuclepore Corp., Pleasanton, Calif.) and placed between each of the two outer plates and the central section. Two pieces of a Nylon mesh (pore size, 100 μm) were placed on either side of the polycarbonate membrane for reinforcement. Preliminary experiments had shown that, if unprotected, many of the membranes ruptured after only 1 day of being exposed to the environment and that the use of reinforcing mesh did not cause leakage of bacteria or sediment from the chambers. The plates were screwed into place with care being taken not to tear or wrinkle the membrane. The assembled chambers were sterilized by steaming for 1 h, as conventional autoclaving was found to deform them. One hundred fifty grams of sediment was placed in the large chambers, while 80 g of sediment was placed in the small chambers. Control chambers were prepared and filled with autoclaved sediment. The chambers were transported to the sites on ice and then placed in the bottom of a metal cage. A sheet of fiberglass mesh was used for additional protection of the chambers and to keep them in place.

The chambers (approximately 30) containing freshwater sediment were deployed over two periods (December 1992 to February 1993 and March 1993 to May 1993) at a site in Prospect Creek. During the first Prospect Creek experiment small chambers were used, while in the second experiment the larger chambers were used. The cage was attached by a chain to a structure on the bank of the creek and lowered to the bottom at a depth of 4 m, about 6 m from the creek bank. The chambers (approximately 30) containing marine sediment (M2) were deployed over two periods (December 1992 to March 1993 and March 1993 to April 1993) in Sydney Harbour, just off Point Piper on the south side of the harbor. Large chambers were used for both of the Point Piper experiments. The cage was attached by a chain to a Maritime Services Board mooring and lowered to the bottom at a depth of approximately 16 m.

Some of the sediment collected for the experiments was retained, before the chambers were filled, as the time zero sample. Sampling involved periodic retrieval of the cages and withdrawal of three chambers, from random positions within the cage, from each site. In the first experiment, chambers were withdrawn after 1, 2, 3, 6, 10 or 14 (for Prospect Creek and Point Piper, respectively), 28, and 56 or 58 days (for Prospect Creek and Point Piper, respectively). In the second experiment, chambers were withdrawn for analysis after 1, 2, 3, 7, 14, 28, and 56 (for Prospect Creek and Point Piper, respectively) or 56 and 85 days (for Prospect Creek only). Single control chambers were also periodically withdrawn. Chambers were transported back to the laboratory on ice. A sample of water was taken with a 2-liter Hydro-bios sampler (Apparatebau GmbH, Kiel-Holtenau,

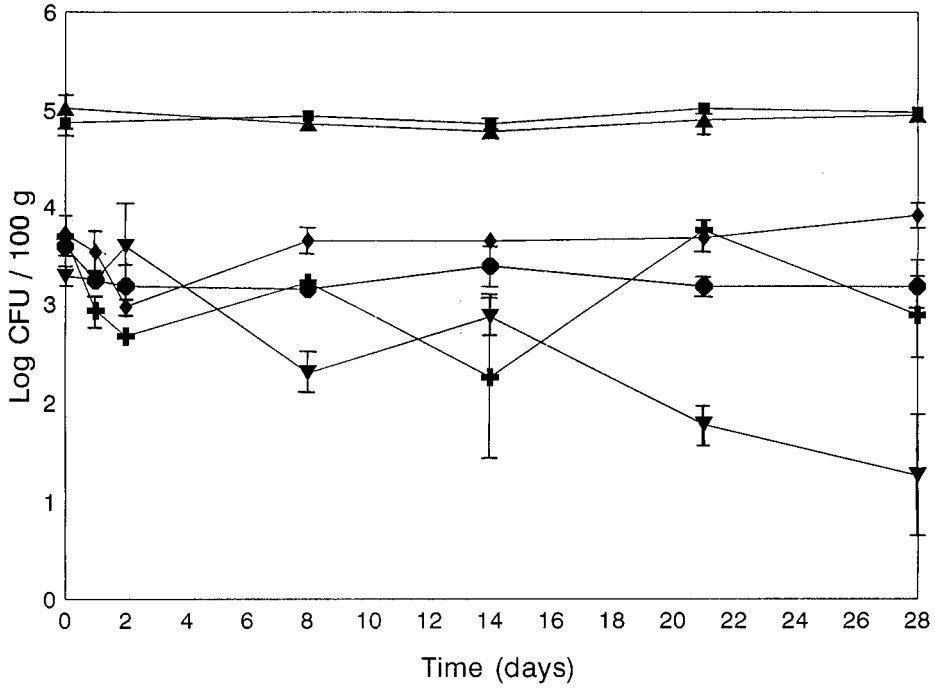

FIG. 2. Effect of predation on bacterial survival in marine sediment (M5). ▲, *C. perfringens*; ■, *C. perfringens* with cycloheximide; ●, fecal streptococci; ◆, fecal streptococci with cycloheximide; ▼, fecal coliforms; ✚, fecal coliforms with cycloheximide. The data are means of two replicate experiments. The error bars represent 1 standard deviation.

Germany) from 1 m above the bottom of the creek or harbor each time sample chambers were retrieved. Temperature, turbidity (Monitek 151 meter; FSE Scientific, Sydney, Australia), pH (pH192 meter; Wissenschaftlich-Technische Werkstatten, Weilheim, Germany), and salinity (LF196 meter; Wissenschaftlich-Technische Werkstatten) of the water samples were measured, and concentrations of total phosphorus, nitrate, ammonia, and organic nitrogen in the water samples were determined (1).

In the laboratory, the three chambers were opened aseptically, and the sediment was removed and placed into three sterile containers. Sediments were pretreated by sonication and analyzed for fecal coliforms, fecal streptococci, *C. perfringens* spores, and total *C. perfringens* as described previously.

VNC survival studies. Closed-bottle microcosms were set up by weighing out 150 g of gamma-irradiated marine sediment (60 kGy from a ^{60}Co source) into each of three sterile containers. Lysis of existing bacterial cells in the wet sediment would be expected to occur at this dose. Starved cultures of an environmental *E. coli* isolate, known to adopt the VNC state, from a saline site of the Georges River, were used to inoculate the three microcosms to a level of about 10^{10} cells per 100 g. A heavy inoculum allows dilutions of the sediment to be made, facilitating microscopic examination. Cells were harvested from nutrient broth and washed by a centrifugation-resuspension procedure (33). After withdrawal of an initial sample for analysis, the microcosms were incubated at 20°C in the dark without agitation. Samples were then withdrawn periodically and pretreated by sonication as described previously, before being analyzed for culturable *E. coli* by membrane filtration using mFC agar. Direct total counts using acridine orange and direct viable counts using acridine orange and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium (INT) chloride (Sigma Chemical Co., St. Louis, Mo.) were carried out by the methods of Hobbie et al. (13) and Quinn (22), respectively. Diluted samples were incubated for 4 h in the dark at room temperature with INT chloride (final concentration, 0.02%), and then acridine orange (final concentration, 0.01%) was added for 5 min. Stained samples were then filtered through black polycarbonate membranes (pore size, 0.2 μm) (Nuclepore) and mounted on microscope slides. The slides were examined by epifluorescence microscopy (Nikon Optiphot-2 microscope with episcopic-fluorescence attachment EF-D) with an oil immersion objective at a magnification of ×1,250. Between 10 and 100 bacteria in 20 fields of view were counted. The total number of cells per field and the number of cells containing dark, optically dense INT-formazan deposits were determined and used to estimate the numbers of total and viable bacteria, respectively, present in the samples. These were easily distinguished from nonbacterial, autofluorescing particles.

Data analysis. When appropriate, the data were analyzed with the SAS statistical software package (SAS Institute).

RESULTS

Predation studies. The survival rates of fecal coliforms, fecal streptococci, and *C. perfringens* spores in the presence and absence of predators in M5 and Whale Beach sediments are illustrated in Fig. 2 and 3, respectively. The survival patterns of *C. perfringens* spores in M5 and Whale Beach sediments were similar in microcosms whether or not cycloheximide was added. The numbers of *C. perfringens* spores remained constant throughout the experiments. In both sediment types there was a slight increase in numbers of fecal coliforms in microcosms containing cycloheximide compared with microcosms without cycloheximide. In Whale Beach sediment there was a greater decrease in numbers of fecal streptococci in microcosms not containing cycloheximide, whereas in M5 sediment the survival characteristics were similar whether or not cycloheximide was added. The results indicate that fecal coliforms and possibly fecal streptococci in sediments may grow in the absence of predators. However, in the presence of predators there is a net decrease in numbers of bacteria recovered.

In situ microcosm survival studies. The survival of fecal coliforms, fecal streptococci, *C. perfringens* spores, and total *C. perfringens* was examined in both marine (M2) and freshwater (Prospect Creek) sediments in two in situ experiments. Initially, experiments were designed to be of a 6-month duration. However, vandalism combined with harsh environmental conditions shortened and varied the duration of the experiments.

In the marine sediment (Fig. 4 and 5) the estimated numbers of *C. perfringens* spores and total *C. perfringens* organisms were generally constant until the last sampling days. Furthermore, spores made up the majority of the *C. perfringens* population in the sediment. The cage containing the chambers was crushed after 58 days in the first experiment and was lost after 28 days

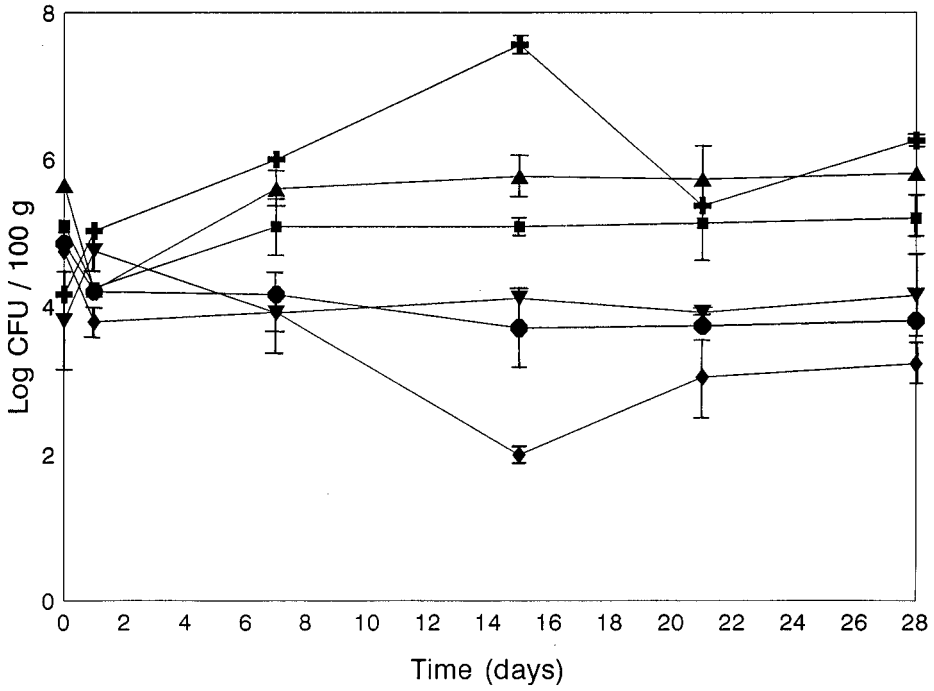

FIG. 3. Effect of predation on bacterial survival in urban run-off-impacted Whale Beach sediment. ▲, *C. perfringens*; ■, *C. perfringens* with cycloheximide; ●, fecal streptococci; ◆, fecal streptococci with cycloheximide; ▼, fecal coliforms; ✚, fecal coliforms with cycloheximide. The data are means of two replicate experiments. The error bars represent 1 standard deviation.

in the second experiment, resulting in the premature termination of both experiments. Throughout the first (Fig. 4) and second (Fig. 5) marine experiments the estimated numbers of fecal coliforms and fecal streptococci decreased gradually with time. The decline in numbers of fecal coliforms was more rapid than the decline in numbers of fecal streptococci. In the first experiment (during Australian summer), numbers both of fecal coliforms and of fecal streptococci decreased by 2 to 3 orders of magnitude. However, in the second experiment (during Australian fall) the numbers of fecal coliforms decreased by 4 orders of magnitude while the numbers of fecal streptococci decreased by only 1 order of magnitude.

In the freshwater sediment (Fig. 6 and 7), the estimated numbers of *C. perfringens* spores and the total numbers re-

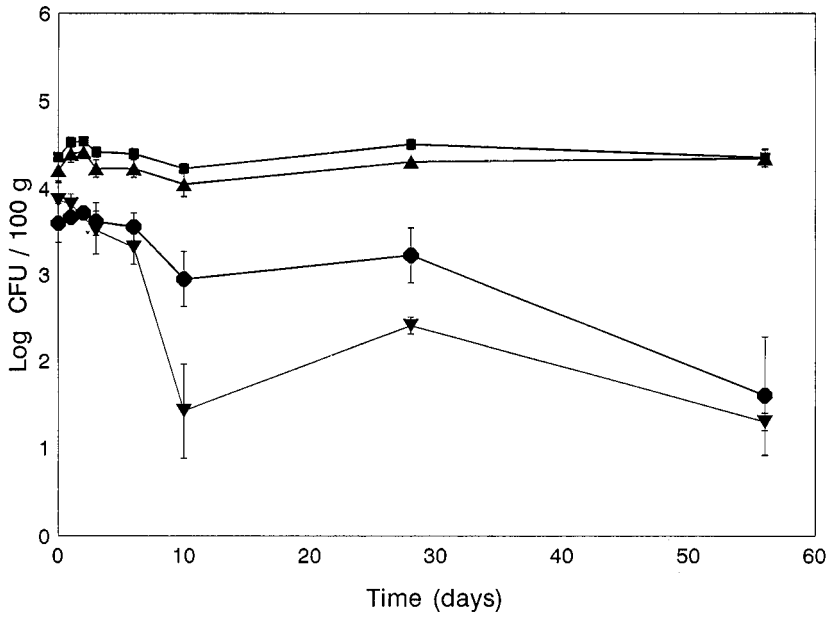

FIG. 4. In situ survival of bacterial in marine sediment (M2) in microcosms (experiment 1). ■, total *C. perfringens*; ▲, *C. perfringens* spores; ●, fecal streptococci; ▼, fecal coliforms. The data are means of six counts, i.e., duplicate counts from three chambers. The error bars represent the 95% confidence intervals of the means.

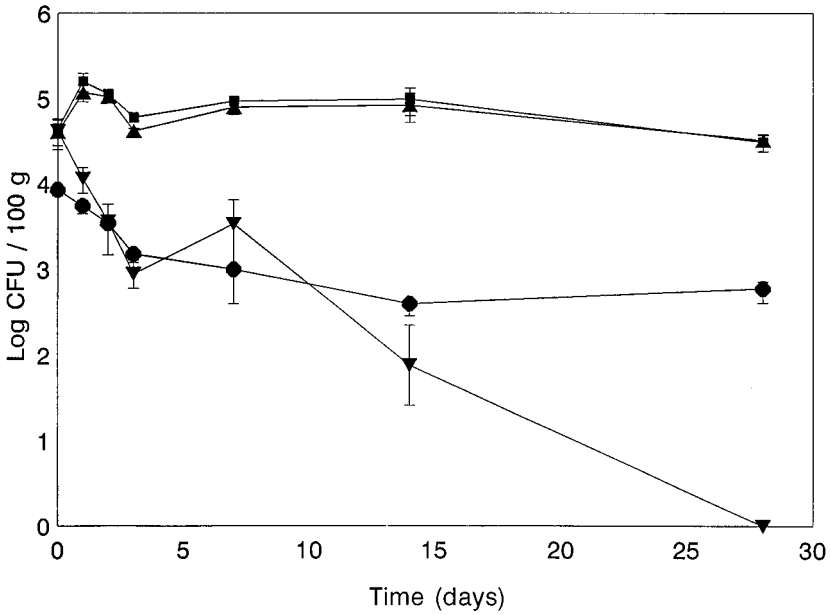

FIG. 5. In situ survival of bacteria in marine sediment (M2) in microcosms (experiment 2). ■, total *C. perfringens*; ▲, *C. perfringens* spores; ●, fecal streptococci; ▼, fecal coliforms. The data are means of six counts, i.e., duplicate counts from three chambers. The error bars represent the 95% confidence intervals of the means.

mained constant until the last sampling days. Again, spores made up the majority of the *C. perfringens* population. As in the marine experiments, there was a gradual decrease with time in estimated numbers of fecal coliforms and fecal streptococci. The decline in numbers of fecal coliforms was more rapid than the decline in numbers of fecal streptococci. In both the first (Fig. 6) and the second (Fig. 7) experiments the numbers of fecal streptococci decreased by approximately 1 order of magnitude. The numbers of fecal coliforms, however, reduced by 2 to 3 orders of magnitude in the first experiment and approximately 5 orders of magnitude in the second experiment. The first and second experiments at Prospect Creek were discontinued after 56 and 85 days, respectively, as a result of the loss of the cage. Generally, no organisms or very small numbers of organisms were detected in the control chambers containing sterile sediment, and it was thought that the few control chambers which did contain bacteria were contaminated during opening to remove the sediment for analysis.

The equation commonly fitted to bacterial decay is $N/N_0 = e^{-bt}$ (6), which is mathematically equivalent to $\log_{10}(N/N_0) =$

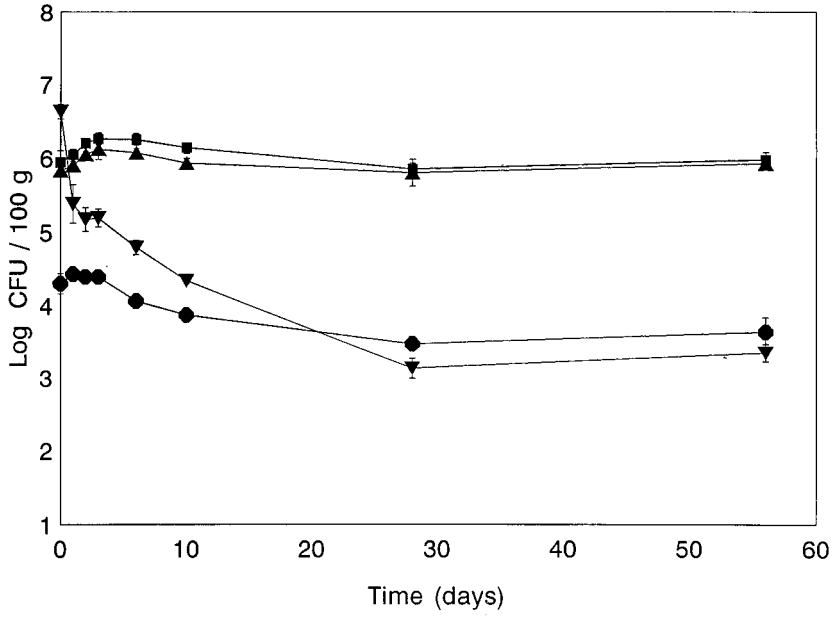

FIG. 6. In situ survival of bacteria in freshwater sediment in microcosms (experiment 1). ■, total *C. perfringens*; ▲, *C. perfringens* spores; ●, fecal streptococci; ▼, fecal coliforms. The data are means of six counts, i.e., duplicate counts from three chambers. The error bars represent the 95% confidence intervals of the means.

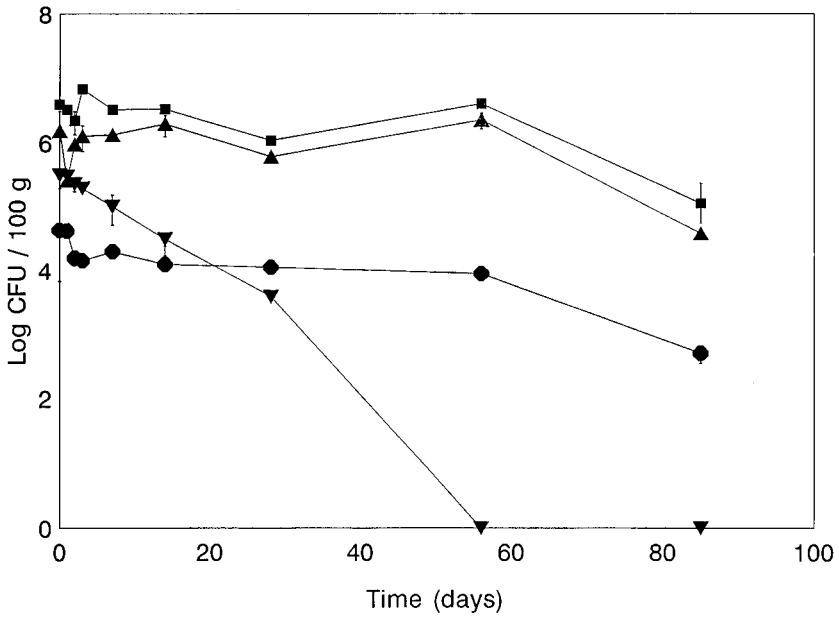

FIG. 7. In situ survival of bacteria in freshwater sediment in microcosms (experiment 2). ■, total *C. perfringens*; ▲, *C. perfringens* spores; ●, fecal streptococci; ▼, fecal coliforms. The data are means of six counts, i.e., duplicate counts from three chambers. The error bars represent the 95% confidence intervals of the means.

$-bt$, where N is the count at time t, N_0 is the count at time zero, and b is the decay rate constant. Expressed in this form, the data should fit a straight line when log count is graphed against days. However, the fecal coliform data graphed in this form appeared to exhibit curvature, indicating that the usual exponential decay equation was inappropriate for the decay of fecal coliforms in sediments. To formally test for curvature, the usual exponential decay model was fitted to the data with quadratic and linear terms in the time variable and tested for goodness of fit. Analysis of variance (Table 1) showed that, for fecal coliforms, *C. perfringens* spores, and total *C. perfringens* bacteria, the quadratic term in time was required and, thus, the usual decay model was inadequate. However, for fecal streptococci the usual decay model was adequate.

The results in Table 2 show the physical and chemical characteristics of the water samples taken at the time of sampling. Regression analysis of these data showed that no parameter was significantly related to decay of the bacteria in the chambers (data not shown). Figure 8 shows the relative size distributions of particles in the Malabar and Prospect Creek sediments used in the membrane diffusion chamber experiments. Marine sediment from Malabar contained a greater percentage of larger particles than the freshwater sediment from Prospect Creek, which was made up predominantly of silt and clay.

TABLE 1. Analysis of variance for fitting the usual exponential decay model with linear and quadratic terms in time-variable-to-survival data[a]

Source of variation (time)	FC		FS		CP		TCP	
	SS	*P*	SS	*P*	SS	*P*	SS	*P*
Linear	51.91	0.0001*	12.46	0.0001*	1.02	0.0033*	1.73	0.0019*
Quadratic	24.44	0.0004*	0.63	0.1323	1.93	0.0003*	1.18	0.0066*
Residual	8.96		2.90		0.91		1.32	

[a] FC, fecal coliforms; FS, fecal streptococci; CP, *C. perfringens* spores; TCP, total *C. perfringens*; SS, sum of squares; *, significant at the 5% level.

VNC survival studies. The survival of *E. coli* in marine sediment as determined for total, viable, and culturable bacteria is shown in Fig. 9. Total counts, as determined by the acridine orange direct count, remained constant throughout the duration of both experiments. The number of bacteria detected by the viable counting method (INT reduction) decreased only slightly over the duration of the experiment, to less than an order of magnitude below the total number of bacteria, indicating the death of a small proportion of the population. The numbers of bacteria detected by the cultural method were consistently approximately an order of magnitude lower than the numbers of viable cells, indicating no loss of culturability of the population other than that occurring during the resuspension procedure carried out prior to inoculation of the microcosms.

DISCUSSION

In the absence of predators, fecal coliforms may be capable of growth in marine and freshwater aquatic sediments. Under natural conditions, however, where predators are present, a net die-off occurs (18, 19). Our findings in the predation studies confirm these observations. In the present study, a greater difference between concentrations of bacteria in the presence of predators compared with those in the absence of predators was observed for fecal coliforms than for fecal streptococci. This may have been due to the greater predation by protozoan predators on fecal coliforms (19). Large numbers of prey will normally be reduced by predators to an equilibrium density at which the rate of predation will be balanced by the rate of replacement of the prey (19). The ability of bacteria to resist intracellular killing, therefore, will ensure that the equilibrium density is maintained and will also ensure the survival of the predators.

The absence of predators from microcosms did not appear to affect the survival of *C. perfringens* spores in sediments. This would seem to suggest that *C. perfringens* spores are not preyed upon, although it is more likely that it may reflect the selectivity of cycloheximide for certain predators. Cycloheximide is

TABLE 2. Physical and chemical characteristics of water samples taken at Point Piper and Prospect Creek at times of sampling of membrane diffusion chambers[a]

Sample	Concn (per liter) [*n*]				Turb (NTU) [*n*]	pH [*n*]	Temp (°C) [*n*]	Sal (‰) [*n*]
	NH_3N (mg)	NO_3N (mg)	TKN (mg)	PO_4P (μg)				
Point Piper								
Expt 1	0.19 ± 0.11 [5]	<0.01 ± 0 [5]	0.80 ± 0.51 [5]	37 ± 40 [5]	3.2 ± 1.5 [4]	7.9 ± 0.2 [5]	22.1 ± 0.6 [4]	33.8 ± 1.8 [2]
Expt 2	0.01 ± 0 [6]	ND	0.11 ± 0.05 [6]	25 ± 6.0 [6]	7.7 ± 3.8 [6]	8.0 ± 0.2 [7]	23.1 ± 0.6 [7]	35.4 ± 1.2 [7]
Prospect Creek								
Expt 1	0.23 ± 0.05 [4]	0.38 ± 0.10 [4]	1.00 ± 0.21 [4]	72 ± 46 [6]	72 ± 59 [8]	7.3 ± 0.2 [8]	22.7 ± 2.0 [6]	1.6 ± 1.2 [7]
Expt 2	0.08 ± 0.06 [8]	ND	0.81 ± 0.24 [8]	39 ± 25 [8]	27 ± 27 [8]	7.6 ± 0.4 [9]	23.5 ± 3.1 [9]	3.3 ± 1.8 [9]

[a] TKN, Kjeldahl nitrogen; PO_4P, total phosphorus; Turb, turbidity; NTU, nephelometric turbidity units; Sal, salinity; ND, not determined. The data are means ± standard deviations.

generally considered to be effective against a wide range of predators but may not be effective against predators of *C. perfringens* spores. Though effective against flagellate protozoa, cycloheximide is only partially effective against ciliate protozoa (28). This problem may be overcome by the use of a combination of a number of different eukaryote inhibitors. It is also possible that cycloheximide is more effective in some sediments than others. This may explain the inhibition by cycloheximide of predators of fecal streptococci in Whale Beach sediment but not in marine sediment (M5).

The use of membrane diffusion chambers allowed the survival of a population of microorganisms to be monitored over a long period of time. The partially contained nature of the microcosms ensured that there was no additional bacterial input while allowing the microorganisms to respond to changes in the surrounding environment. Although the use of membrane diffusion chambers to determine bacterial decay rates in water has been criticized because the conditions within the chambers are slow to reflect changes in conditions outside because of slow passage of molecules across the membrane (29), their use was not considered inappropriate for sediment survival studies in which low decay rates are anticipated and rapid responses to changes in the characteristics of the overlying water are unlikely.

The close similarity between the numbers of spores and the total number of *C. perfringens* bacteria in both sediments and the almost identical decay equations for the organisms in two supposedly different states indicate that the majority of the population were spores. The absence of vegetative cells indicated that there was little growth of *C. perfringens* in either of the sediments. The method which was used to obtain a total count of *C. perfringens* was based on the assumption that all of the spores would germinate and grow without being heat shocked and that the vegetative cells would also grow. In the absence of vegetative cells, however, the results showed that this was a reasonable assumption to make, as there was little difference in numbers of spores in heat-shocked and non-heat-shocked portions of the sediments. The only other explanation

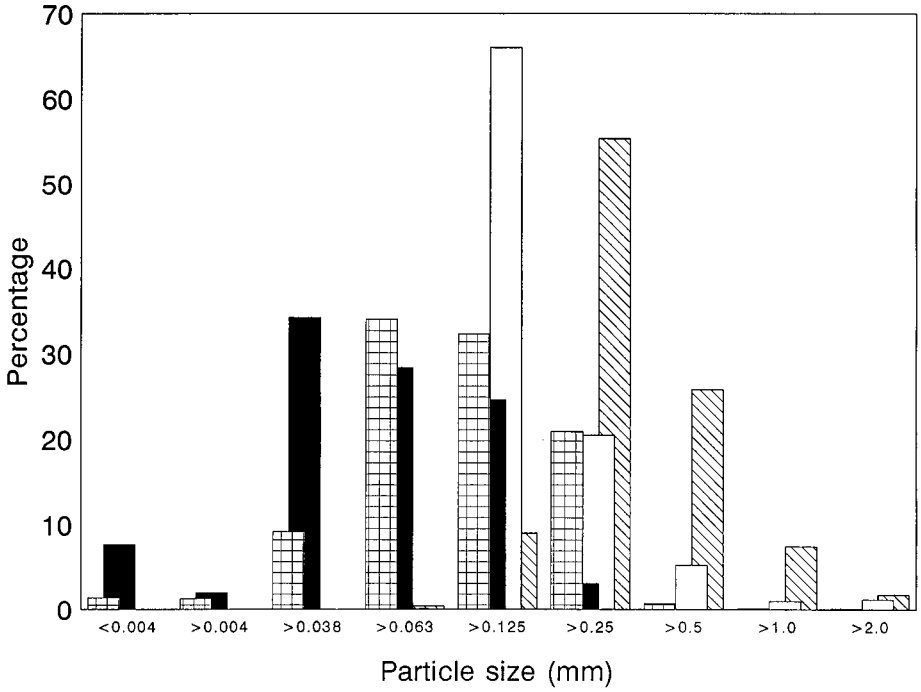

FIG. 8. Particle size distribution of sediment used for in situ survival studies. ⊞ and □, freshwater samples 1 and 2, respectively; ■ and ▨, marine samples 1 and 2, respectively.

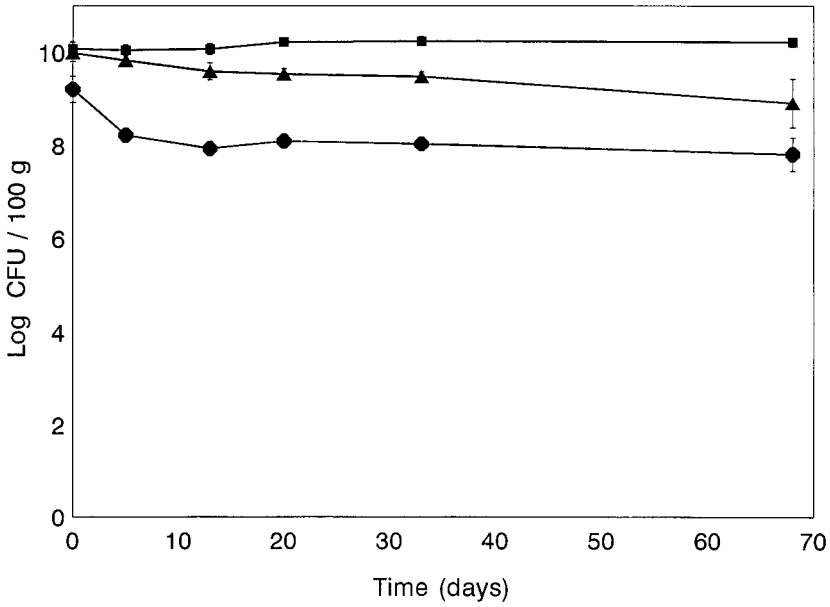

FIG. 9. VNC *E. coli* in marine sediment. ■, total counts; ▲, viable counts (INT reduction); ●, culturable counts. The data are means of counts from triplicate microcosms. The error bars represent the 95% confidence intervals of the means.

would be that the vegetative cells were unable to grow on the chosen medium. *C. perfringens* spores may persist in sediments for an undetermined time whereas other indicator bacteria, such as fecal coliforms and fecal streptococci, decay more rapidly, though their survival is extended compared with that in the water column (18, 25). Hence, spores of *C. perfringens* represent a most conservative indication of sewage presence which correlates well with the fecal sterol, coprostanol (21).

The fact that there was an overall die-off of fecal coliforms and fecal streptococci in the chambers indicates an imbalance between predation and growth, with predation having the more dominant effect. The usual exponential decay model is inappropriate for fecal coliforms and perhaps *C. perfringens*. The survival of these organisms would possibly be better described by models not possessing constant half-life properties. Application of the usual exponential decay equation to the fecal coliform data appears to be confounded by the complex relationship between growth and predation.

Had the numbers of fecal coliforms and streptococci in the membrane diffusion chambers remained at constant levels throughout the experiment, it could be attributed to a counterbalance in numbers due to a decrease by predation and an increase by growth. However, the fact that there was an overall die-off of fecal coliforms and fecal streptococci in the membrane diffusion chambers does not explain the maintenance of high concentrations of these bacteria in inshore sediments after commissioning of the deepwater ocean outfall (2). This suggests that, although the concentrations of bacteria in Malabar sediments may be decreasing because of bacterial die-off, as shown in the membrane diffusion chambers, for the levels to have remained constant following commissioning of the Malabar deepwater ocean outfall there may be an additional input of fecal bacteria from unidentified sources. The most likely explanation is that, during and following storm activity, particle-bound microorganisms are carried through the harbor mouths into the coastal waters. Mathematical modelling has predicted that, during severe storms, up to 70% of the sewage being carried by the sewerage system is discharged through overflows into rivers and estuaries through the mouths of Sydney's three major harbors during the first 24 h (32). However, this is based on a 5-year recurrence interval. Sewer overflows into coastal storm water drains occur more frequently and may be a more obvious source of fecal microorganisms.

Gram-negative bacterial cells introduced into seawater lose their abilities to form colonies within a short period, though they may still be detected at fairly high levels by viable counting methods (33). Our study showed that this was not the case with bacterial cells introduced into marine sediments, which appeared to remain culturable at fairly high concentrations for the full duration of the experiment (68 days). The difference in the numbers of bacteria detected by the cultural method and the numbers detected by the viable counting method did not change over time. This observation indicates that storage of the bacteria in marine sediment does not result in any further loss of the ability to form colonies by the viable population. The observed initial difference between the numbers of bacteria detected by the cultural method and the numbers of bacteria detected by the viable counting method in the sample taken at time zero may be a result of the centrifugation-resuspension procedure used to prepare the seed bacterial culture, as this procedure involves resuspension in sterile seawater or freshwater low in nutrients. This will result in the loss of culturability of a small proportion of the bacteria due to transfer from a nutrient-rich environment to a nutrient-poor environment. The VNC form has been proposed as a survival strategy which some bacteria may employ when unfavorable conditions, particularly low nutrient concentrations, are encountered in the environment (33). As nutrients are often associated with sediment particles, it seems likely that the nutrients available to bacteria in sediments would be plentiful and, therefore, a significant proportion of the cells are unlikely to adopt a VNC form in sediments. Indeed, growth and predation are more likely.

ACKNOWLEDGMENTS

We thank the technical staff of AWT, EnSight, for their assistance in the field.

We thank the Water Board for financing the study with assistance from the Special Environmental Levy.

REFERENCES

1. **American Public Health Association.** 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington, D.C.
2. **Ashbolt, N. J., G. S. Grohmann, and C. Kueh.** 1993. Significance of specific bacterial pathogens in the assessment of polluted receiving waters of Sydney. Water Sci. Technol. **27:**449–452.
3. **Australian Water Technologies, EnSight.** 1993. Microbiology test methodologies. Australian Water Technologies, EnSight, West Ryde, Australia.
4. **Bitton, G., Y. Henis, and N. Lahav.** 1972. Effect of several clay minerals and humic acid on the survival of *Klebsiella aerogenes* exposed to ultraviolet irradiation. Appl. Microbiol. **23:**870–874.
5. **Chamberlin, C. E., and R. Mitchell.** 1978. A decay model for enteric bacteria in natural waters, p. 325–348. *In* R. Mitchell (ed.), Water pollution microbiology, vol. 2. Wiley, New York.
6. **Chick, H.** 1908. Investigation of the law of disinfection. J. Hyg. **8:**92–158.
7. **Department of the Environment, Department of Health and Social Security, and Public Health Laboratory Service.** 1983. The bacteriological examination of drinking water supplies 1982. Methods for examination and associated materials. Reports on health and medical subjects no. 71. Her Majesty's Stationary Office, London.
8. **Enzinger, E. M., and R. C. Cooper.** 1976. Role of bacteria and protozoa in the removal of *Escherichia coli* from estuarine waters. Appl. Environ. Microbiol. **31:**758–763.
9. **Gauthier, M. J., P. M. Munro, and V. A. Breittmayer.** 1989. Influence of prior growth conditions on low nutrient response of *Escherichia coli* in seawater. Can. J. Microbiol. **35:**379–383.
10. **Gerba, C. P., and J. S. McLeod.** 1976. Effect of sediments on the survival of *Escherichia coli* in marine waters. Appl. Environ. Microbiol. **32:**114–120.
11. **Ghoul, M., T. Bernard, and M. Cormier.** 1986. Evidence that *Escherichia coli* accumulates glycine betaine from marine sediment. Appl. Environ. Microbiol. **56:**551–554.
12. **Grohmann, G. S., N. J. Ashbolt, M. Genova, G. Logan, P. Cox, and C. Kueh.** 1993. Detection of viruses in coastal and river water systems in Sydney, Australia. Water Sci. Technol. **27:**457–462.
13. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.
14. **Hood, M. A., and G. E. Ness.** 1982. Survival of *Vibrio cholerae* and *Escherichia coli* in estuarine waters and sediments. Appl. Environ. Microbiol. **43:**578–584.
15. **Jannasch, H. W., and G. E. Jones.** 1959. Bacterial populations in seawater as determined by different methods of enumeration. Limnol. Oceanogr. **4:**128–139.
16. **Jones, G. E.** 1964. Effect of chelating agents on the growth of *Escherichia coli* in seawater. J. Bacteriol. **87:**484–499.
17. **Kuzhinovskii, V. A., I. N. Mitskevich, and M. N. Poglazova.** 1991. A method for counting microorganisms in marine bottom sediments. Mikrobiologiya **60:**377–382.
18. **LaLiberte, P., and D. J. Grimes.** 1982. Survival of *Escherichia coli* in lake bottom sediment. Appl. Environ. Microbiol. **43:**623–628.
19. **Marino, R. P., and J. J. Gannon.** 1991. Survival of faecal coliforms and faecal streptococci in storm drain sediment. Water Res. **25:**1089–1098.
20. **McDaniel, J. A., and D. G. Capone.** 1985. A comparison of procedures for the separation of aquatic bacteria from sediments for subsequent direct enumeration. J. Microbiol. Methods **3:**291–302.
21. **Nichols, P. D., R. Leeming, M. S. Rayner, V. Latham, N. J. Ashbolt, and C. Turner.** 1993. Comparison of the abundance of the fecal sterol coprostanol and fecal bacterial groups in inner-shelf waters and sediments near Sydney, Australia. J. Chromatogr. **643:**189–195.
22. **Quinn, J. P.** 1984. The modification and evaluation of some cytochemical techniques for the enumeration of metabolically active heterotrophic bacteria in the aquatic environment. J. Appl. Bacteriol. **57:**51–57.
23. **Perez-Rosas, N., and T. C. Hazen.** 1988. In situ survival of *Vibrio cholerae* and *Escherichia coli* in tropical coral reefs. Appl. Environ. Microbiol. **54:**1–9.
24. **Pike, E. B., A. H. L. Gameson, and D. J. Gould.** 1970. Mortality of coliform bacteria in seawater samples in the dark. Rev. Int. Oceanogr. Med. **18/19:** 97–107.
25. **Pommepuy, M., J. F. Guillaud, E. Dupray, A. Derrien, F. Le Guyader, and M. Cormier.** 1992. Enteric bacteria survival factors. Water Sci. Technol. **12:**93–103.
26. **Roper, M. M., and K. C. Marshall.** 1979. Effects of salinity on sedimentation and of particulates on survival of bacteria in estuarine habitats. Geomicrobiol. J. **1:**103–116.
27. **Roszak, D. B., D. J. Grimes, and R. R. Colwell.** 1984. Viable but non-culturable *Salmonella enteritidis* in aquatic systems. Can. J. Microbiol. **30:** 334–338.
28. **Sherr, B. F., E. B. Sherr, T. L. Andrew, R. D. Fallon, and S. Y. Newell.** 1986. Trophic interactions between heterotrophic protozoa and bacterioplankton in estuarine water analysed with selective metabolic inhibitors. Mar. Ecol. Prog. Ser. **32:**169–179.
29. **Springthorpe, V. S., C. L. Loh, W. J. Robertson, and S. A. Sattar.** 1993. *In situ* survival of indicator bacteria, MS-2 phage and human pathogenic viruses in river water. Water Sci. Technol. **27:**413–420.
30. **Van Donsel, D. J., and E. E. Geldreich.** 1971. Relationship of salmonellae to fecal coliforms in bottom sediments. Water Res. **5:**1079–1087.
31. **Velji, M. I., and L. T. Albright.** 1986. Microscopic enumeration of attached marine bacteria in seawater, marine sediment, fecal matter, and kelp blade samples following pyrophosphate and ultrasound treatments. Can. J. Microbiol. **32:**121–126.
32. **Water Board (Sydney, Australia).** Unpublished data.
33. **Xu, H. S., N. Robert, F. L. Singleton, R. W. Attwell, D. J. Grimes, and R. R. Colwell.** 1982. Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb. Ecol. **8:**313–323.