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# Distribution of Indicator Bacteria and *Vibrio parahaemolyticus* in Sewage-Polluted Intertidal Sediments

M. P. SHIARIS,[1]* A. C. REX,[2] G. W. PETTIBONE,[2]† K. KEAY,[1] P. McMANUS,[1] M. A. REX,[1] J. EBERSOLE,[1] AND E. GALLAGHER[2]

*Department of Biology[1] and Environmental Sciences Program,[2] University of Massachusetts–Boston Harbor Campus, Boston, Massachusetts 02125*

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**The impact of a sewage point source on the bacterial densities in an intertidal mud flat in Boston Harbor, Mass., was investigated. The area, Savin Hill Cove, acts as a receiving basin for a combined storm and sewage outlet (CSO). Preliminary examination of sediments and overlying water at high tide demonstrated that fecal coliforms were present in sediments at abundances 2 to 4 orders of magnitude higher than in the overlying water column. The following bacterial counts were determined from sediments along a sampling transect extending 460 m from the CSO: total bacteria by epifluorescent microscopy, heterotrophic bacteria by plate counts on nutrient-rich and nutrient-poor media, fecal coliforms and enterococci by membrane filtration, and *Vibrio parahaemolyticus* by a most-probable-number technique with a resuscitation step. Median sediment grain size, average tidal exposure, carbon/nitrogen ratio, and total organic carbon were also measured. All bacterial indices, except for *V. parahaemolyticus*, declined significantly with distance from the outfall. Multiple regression analysis indicated that tidal exposure (low tides) may affect densities of total bacteria. Fecal coliforms and enterococci were still present in appreciable numbers in sediments as far as 460 m away from the CSO. In contrast, *V. parahaemolyticus* densities did not correlate with the other bacterial counts nor with any of the environmental parameters examined. These results indicate that intertidal sediments which adjoin point sources of pollution are severely contaminated and should be considered as potentially hazardous reservoirs of sewage-borne diseases.**

Coastal mudflats are important habitats for a variety of intertidal species, including commercially important shellfish. Many coastal communities have a long history of disposing sewage onto tidal flats, thereby contaminating shellfish and necessitating monitoring by government agencies to protect public health. The primary bacteriological indicators used to assess the safety of shellfish beds are total coliform counts or fecal coliform counts of overlying waters. However, seafood poisoning resulting from consumption of contaminated shellfish continues to be a substantial problem, pointing up the inadequacy of coliform counts in seawater as a measure of public health risk. This use of coliform counts has been questioned because fecal coliforms die off rapidly in seawater (3, 6); because they are poor indicators of bacteria indigenous to estuaries, such as pathogenic vibrios (10); and because low coliform counts belie the possible presence of enteric viruses (7). Enterococci have been suggested as an alternative to fecal coliforms because they survive longer in seawater (2, 5, 13), but they still would not adequately indicate the presence of viruses or vibrios.

While coliform and enterococci counts in seawater have a number of recognized shortcomings, very little is known about the abundance and distribution of these bacteria in an important shellfish habitat: intertidal sediments. Subtidal sediments (and freshwater sediments) harbor fecal indicator bacteria (11, 12, 15, 18) at much higher densities than in the water column, but to our knowledge, the distribution of indicator bacteria in intertidal sediments has not been reported. In contrast to subtidal sediments, intertidal mudflats are subject to daily extremes of light, temperature, and wet-dry cycles. Sewage outfalls are often located near intertidal mudflats that support shellfish beds.

We investigated the impact of a point source of sewage, a combined storm and sewage overflow (CSO) in Boston Harbor, Mass., on the bacterial numbers in sediments of an intertidal mudflat. We relate densities of fecal indicators and *Vibrio parahaemolyticus* to distance from the outfall and physical characteristics of the sediments.

## MATERIALS AND METHODS

**Study area.** Savin Hill Cove is located in the Dorchester Bay section of Boston Harbor (Fig. 1). At low tide, much of the cove is exposed as an intertidal mud flat. Raw sewage enters the cove from the Fox Point CSO, which is the largest CSO in Dorchester Bay, having a 3.7- by-6.1-m feeder main located below the low tide mark. A sampling transect was established beginning at the mouth of the CSO and extending 460 m northwest (Fig. 1). The transect was intended to represent a gradient of sewage pollution and tidal exposure. The L-shape of the transect was necessitated by the presence of a sandbar which extends from Fox Point northwest of sites 1 to 4. Surficial sediments were collected at 15 sites along this transect. Sites 1 to 3 were subtidal, and sites 4 to 15 were intertidal.

**Sample collection.** Sediment samples along the transect were collected during low tide on 5 June 1985. During an earlier preliminary survey (15 and 17 October 1984), sites 2, 4, 7, and 13 were sampled at high tide for both sediments and overlying water.

To collect intertidal sediment samples, the top 0.5 cm (oxidized layer) of sediment was aseptically scraped from a 400-$cm^2$ area into a sterile glass bottle. At high tide, sediment samples were taken from a small motorboat with an Ekman grab, and ca. 50 g of the oxidized surface layer was asepti-

* Corresponding author.
† Present address: Department of Biology, State University College at Buffalo, Buffalo, NY 14222.

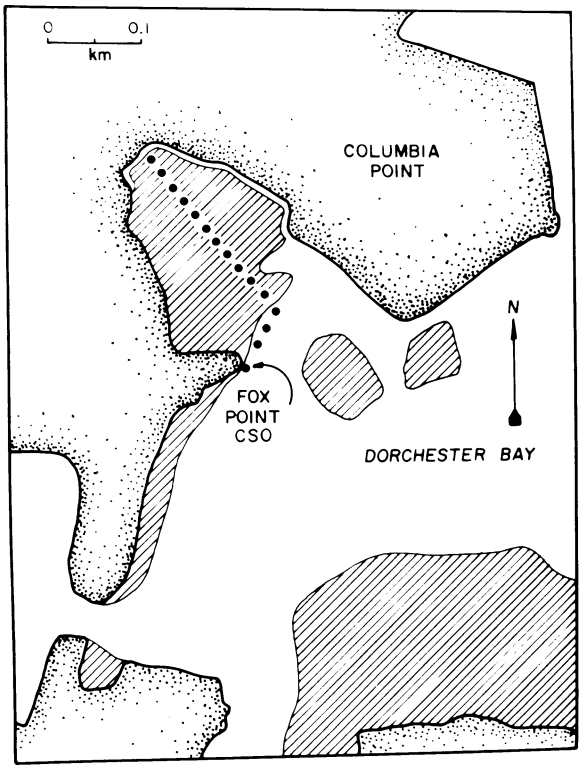


FIG. 1. Map of Savin Hill Cove, Boston Harbor, Mass., with transect sampling sites.

cally subsampled. In addition, water samples were taken with a sterile glass bottle at the surface after the bottle was rinsed twice with surface water. All samples were processed in the laboratory within 1 h of collection. Aliquots for particle size measurement, organic carbon, and carbon/nitrogen (C/N) ratio analyses were frozen at −10°C.

**Bacterial enumeration.** Sediment from each site was diluted 1:10 (wet wt/vol) in autoclaved estuarine water and homogenized in a Waring blender for 20 s. These slurries were used to prepare all the cultures and the total direct counts. Triplicate sediment aliquots were dried in aluminum weighing tins at 105°C to determine sediment dry weight.

Membrane filtration procedures were used for enumeration of fecal coliforms and enterococci in the transect sediment samples. Homogenized sediment was serially diluted in sterile 0.1% (wt/vol) peptone water, and appropriate dilutions were filtered through 0.45-μm-pore-size membranes (47-mm diameter; Gelman Sciences, Inc., Ann Arbor, Mich.) and then rinsed twice with peptone water. Three replicate filters were prepared for each dilution. Subsequent processing of samples for fecal indicators was by standard methods (1).

To enumerate fecal coliforms, we placed filters in 50-mm culture dishes on MF support pads (Millipore Corp., Bedford, Mass.) saturated with mFC broth supplemented with 1% (wt/vol) rosolic acid (Fisher Scientific Co., Pittsburgh, Pa). Dishes were incubated for 22 to 26 h submerged in a circulatory water bath at 44.5 ± 0.2°C. All typical blue colonies were counted.

To enumerate enterococci, we placed filters on KF streptococcal agar supplemented with 0.1% (wt/vol) triphenyltetrazolium chloride (Sigma Chemical Co., St. Louis, Mo.) and incubated them for 45 to 51 h at 35 ± 0.5°C. Typical red colonies were counted.

Heterotrophic plate counts were obtained by the spread plate method on plate count agar and on dilute estuarine agar (DEA). In preliminary experiments, DEA yielded the highest plate counts of sediment bacteria when compared against several commonly used marine and estuarine plating media. The composition of DEA was: peptone, 0.1 g/liter; yeast extract, 0.1 g/liter; $FeCl_3$, 60 mg/liter; agar, 18 g/liter; aged, filtered (through a 0.2-μm-pore-size Nuclepore [Pleasanton, Calif.] membrane filter) estuarine water, 500 ml; and distilled water, 500 ml. Each sample was diluted as above, plated in triplicate, and incubated at room temperature (23 to 25°C). Colonies were counted on the plate count agar after 48 h of incubation and on the DEA after 1 week of incubation.

All colony counts were done with the aid of a dissecting microscope at ×7 to ×12 magnification. The mean counts of plates showing 30 to 300 colonies were used in the analyses.

The 4′,6-diamidino-2-phenylindole fluorescing stain as described by Porter and Feig (14) was used for the determination of total direct counts. Appropriate peptone water dilutions of sediment slurry were filtered through black Nuclepore membrane filters (25-mm diameter; 0.2-μm pore size) and stained with a 0.1-μg/ml solution of 4′,6-diamidino-2-phenylindole (final concentration). Three replicate filters were prepared for each dilution. At least 15 microscope fields and 300 bacteria were counted per filter. Mean counts were used in the analyses.

For the preliminary samples, taken in October 1984, a five-tube most-probable-number (MPN) technique was used to enumerate fecal coliforms in water and sediment. For water samples, a lauryl tryptose broth tube series was inoculated and incubated at 35°C. All tubes displaying visible turbidity after 24 h were transferred to tubes of EC medium (Difco Laboratories, Detroit, Mich.) and incubated at 44.5°C. The presence of fecal coliforms in EC medium tubes was confirmed by streaking broth cultures on eosin-methylene blue agar plates. Typical lactose-fermenting colonies on eosin-methylene blue agar which were gram-negative rods, indole positive, methyl red positive, Voges-Proskauer negative, and citrate negative were called fecal coliforms. MPNs from sediment were obtained by the same method, using peptone water dilutions of sediment-estuarine water homogenate as the inocula.

A three-tube MPN enrichment technique was used to enumerate *V. parahaemolyticus* in sediment samples. Approximately 50 g of surficial sediment from each site was stirred thoroughly with a glass rod. Aliquots of 10, 1.0, and 0.1 g (wet weight) of sediments were inoculated, in triplicate, into 90, 10, and 10 ml of sterile alkaline peptone broth, respectively. The formulation of alkaline peptone broth was: peptone, 10 g/liter; NaCl, 10 g/liter; pH 8.5. These enrichments were incubated at 25°C for 4 h (resuscitation step); then at 35°C for 18 h (16). After incubation, all enrichments were streaked onto thiosulfate–citrate–bile salts agar plates and the plates were incubated overnight at 35°C. Green colonies were subcultured to new thiosulfate–citrate–bile salts agar plates and plates made from Marine Broth 2216 (Difco) for further isolation and identification.

*V. parahaemolyticus* colonies were identified by the API 20E system (Analytab Products, Plainview, N.Y.) with a probability matrix for the identification of environmental vibrios published by Dawson and Sneath (4). Strips were inoculated, incubated, and read according to the directions of the manufacturer, except that sterile 1% NaCl (wt/vol) instead of water was used to prepare the initial suspension of

bacteria. Organisms with the following characteristics were called *V. parahaemolyticus*: gram-negative rods, green on thiosulfate-citrate-bile salts agar, oxidase positive, oxidation-fermentation-glucose positive for oxidation and fermentation, β-galactosidase negative, arginine dihydrolase variable, lysine decarboxylase variable, ornithine decarboxylase variable, citrate negative, $H_2S$ negative, urease variable, tryptophan deaminase negative, indole positive, Voges-Proskauer negative, gelatin variable, glucose positive, mannitol positive, inositol negative, sorbitol negative, rhamnose negative, sucrose negative, melibiose negative, and arabinose variable. A standard MPN table (1) was used to translate the number of enrichment cultures yielding *V. parahaemolyticus* into MPNs.

All bacteriological media were purchased from Difco Laboratories, Detroit, Mich.

**Physical and chemical parameters.** Median sediment grain size was determined by wet-sieving sediment through a 63-μm mesh sieve. The fraction retained on the sieve was dried and shaken through nested sieves of decreasing mesh size. The fraction that passed through the 63-μm mesh sieve was subjected to pipette sedimentation analysis. The dry weight of each size class was determined by gravimetric analysis, and the median grain size was extrapolated from a cumulative percent-composition graph. The Wentworth scale was used to express grain size in phi (ϕ): phi = $-\log_2 E$, where $E$ is the grain or particle diameter in millimeters (19).

The average tidal exposure (minutes per tidal cycle) was determined by averaging the exposure of each site during a range of tidal amplitudes, including two spring tides and two neap tides.

Both C/N ratios and total organic carbon were determined on a CHN analyzer by the Darling Marine Laboratory, Walpole, Maine.

**Statistical analyses.** Correlation and regression analyses were done on an IBM-PC/XT microcomputer, using the STATPACK statistical package.

## RESULTS AND DISCUSSION

Examination of water and surficial sediment samples from four sites in Savin Hill Cove indicated that fecal coliforms accumulate in sediments at very high levels—2 to 4 orders of magnitude higher than in the overlying water column at high tide (Table 1). The marked decline in sediment coliform densities with increased distance from the outfall implicates the CSO pipe as a major source of coliforms in neighboring sediments. Fecal coliform counts from the pipe, taken at a later date, were $2 \times 10^5$ CFU/100 ml.

A more complete picture of bacterial contamination of sediments along the sampling transect is given in Fig. 2. Total direct counts, plate counts with dilute and rich medium, fecal indicators, and *V. parahaemolyticus* are plotted against distance from the CSO. All counts, with the exception of *V. parahaemolyticus*, decreased with distance from the outfall, declining precipitously along the three subtidal sites closest to the outfall, and then declining more gradually or leveling off with distance across the intertidal area. Curvilinear regressions (of the form $Y = A + B\ln X$) of bacterial counts on distance from the CSO are highly significant ($r$ values range from $-0.82$ to $-0.98$, df = 13, $P < 0.01$), except for *V. parahaemolyticus* ($r = 0.13$, $P > 0.10$). The elevated plate counts near the CSO obtained on dilute medium (DEA) suggest that the outfall augments nutrients for the autochthonous estuarine flora. The high densities on rich medium (plate count agar) probably reflect the introduction of an allochthonous component from the CSO. The latter group dropped off rapidly with distance and reached levels considerably below those of the dilute medium group. Densities of fecal indicators, direct measures of the influence of the outfall, declined with distance from the CSO, but they were still detectable at the farthest station 460 m away. The distribution of *V. parahaemolyticus* appeared to be independent of both distance from the outfall and indicator groups.

TABLE 1. Fecal coliforms in water column and sediments of Savin Hill Cove at high tide

| Site | Date | Fecal coliform MPN (counts/100 ml) | |
|---|---|---|---|
| | | Water | Sediment |
| 2 | 10/15/84 | <20 | 33,000 |
| | 10/17/84 | 2 | 60,000 |
| 4 | 10/15/84 | <20 | 3,300 |
| | 10/17/84 | 7 | 4,900 |
| 7 | 10/15/84 | <20 | 500 |
| | 10/17/84 | 2 | 800 |
| 13 | 10/15/84 | 20 | 200 |
| | 10/17/84 | 5 | 500 |

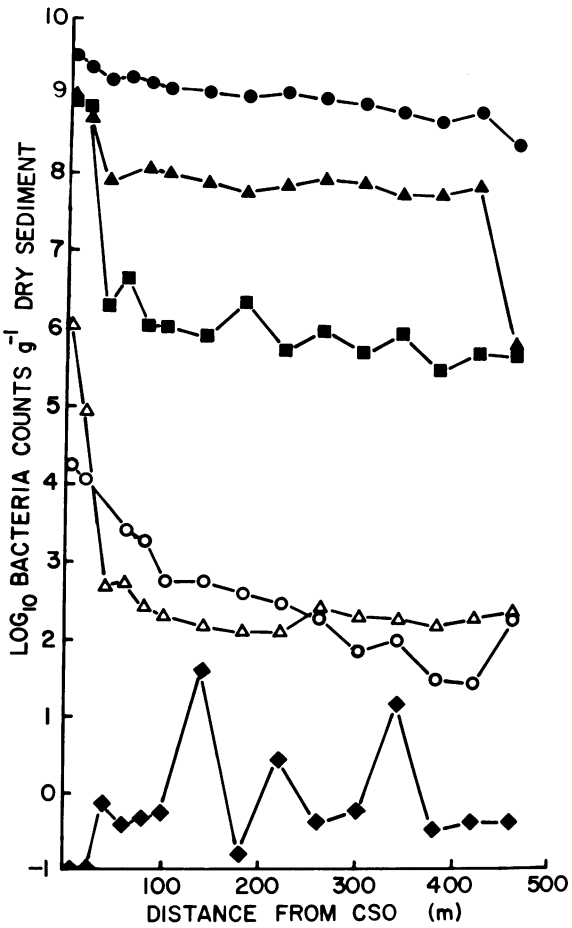


FIG. 2. Bacteria counts in surficial sediments of Savin Hill Cove transect sites. Symbols: ●, total direct microscopic counts; ▲, total plate counts on DEA; ■, total plate counts on standard plate count agar; ○, fecal coliforms; △, enterococci; ◆, *V. parahaemolyticus* MPNs. Sediments were sampled at low tide.

TABLE 2. Physical characteristics of Savin Hill Cove sediments

| Site | Distance from outfall (m) | Avg tidal exposure (min) | Mean particle size (mm) | Total organic carbon (mg $g^{-1}$)[a] |
|---|---|---|---|---|
| 1 | 5 | 0 | ND[b] | ND |
| 2 | 20 | 0 | ND | ND |
| 3 | 40 | 0 | ND | ND |
| 4 | 60 | 25 | 0.0090 | 49.08 |
| 5 | 80 | 39 | 0.0118 | ND |
| 6 | 100 | 85 | 0.0884 | 27.10 |
| 7 | 140 | 77 | 0.0385 | 26.39 |
| 8 | 180 | 53 | 0.0192 | 31.62 |
| 9 | 220 | 81 | 0.0156 | 37.55 |
| 10 | 260 | 126 | 0.0179 | 38.80 |
| 11 | 300 | 165 | 0.0254 | 30.06 |
| 12 | 340 | 192 | 0.0313 | 28.60 |
| 13 | 380 | 215 | 0.0272 | 28.17 |
| 14 | 420 | 229 | 0.0237 | 28.97 |
| 15 | 460 | 224 | 0.0335 | 23.18 |

[a] Dry sediment weight.
[b] ND, Not determined.

For the 11 intertidal sites (the 3 sites nearest the CSO were permanently subtidal), bacterial densities were negatively correlated with average tidal exposure, mean particle size of sediments, and carbon-to-nitrogen ratio and positively correlated with total organic carbon. Data for these variables are presented in Table 2. Table 3 is a matrix of correlation coefficients among the environmental variables and bacterial counts.

Multiple regression analyses were performed to determine whether the bacterial counts could be predicted by the environmental variables (Table 4). Tidal exposure accounted for 78% of the variation in total direct count. The C/N ratio enters the equation as a significant subordinate variable explaining 13% of the variance. This is surprising because C/N is uncorrelated by itself with total direct count ($r = -0.25$, $P > 0.05$). C/N significantly correlates with residual variation ($r = -0.71$, $P < 0.05$) after tidal exposure enters the equation, but the goodness of fit is attributable to two outlying points, an unusually positive residual at station 4 and an unusually negative residual at station 8. When these two stations are removed, the regression of C/N on the residuals is insignificant ($r = -0.28$). Thus, its significant entry into the equation appears to be attributable to certain points rather than to any overall trend. The fecal indicators correlate significantly with total organic carbon. In both cases, however, the correlations are due largely to elevated counts and levels of organic carbon at station 4 near the CSO. When station 4 is removed from the analysis, the correlations are no longer significant. Enterococci are not correlated with any other environmental variable, and fecal coliforms are correlated only with tidal exposure (Table 3).

The overall picture to emerge for the intertidal stations is that tidal exposure may affect total direct count and fecal coliforms. Total organic carbon may influence the indicators close to the outfall, but beyond station 4 (60 m) there is no clear relationship. The pattern of abundance in *V. parahaemolyticus* appears to be independent of other bacterial counts and the environmental parameters that were measured (Table 3). *V. parahaemolyticus* was found at all the sites except for the two closest to the outfall. The distribution of *V. parahaemolyticus* was patchy, but it can clearly reach appreciable numbers in intertidal sediment ($10^3$/100 g [wet weight] at site 7).

Distance from the outfall is highly correlated with total direct count ($r = -0.94$, $P < 0.001$) over the span of intertidal stations considered in the multiple regression analysis. Distance from outfall was not used in the analysis because it does not measure local environmental influence in any direct way and because it is so statistically redundant with tidal exposure ($r = 0.96$, $P < 0.001$). The latter relationship exists because the intertidal region of the sampling transect slopes up from the CSO, which is near shore in an artificially dredged depression. Except for a significant

TABLE 3. Simple correlation matrix for bacterial counts and physical variables for Savin Hill Cove transect[a]

| Variable | TDC | VIB | COLI | ENT | SIZE | TIDE | TOC |
|---|---|---|---|---|---|---|---|
| VIB | −0.06 | | | | | | |
| COLI | 0.82[b] | −0.09 | | | | | |
| ENT | 0.55 | −0.26 | 0.80[b] | | | | |
| SIZE | −0.04 | −0.16 | −0.28 | −0.28 | | | |
| TIDE | −0.90[c] | −0.09 | −0.71[b] | −0.34 | 0.09 | | |
| TOC | 0.71[d] | −0.28 | 0.74[b] | 0.73[b] | −0.54 | −0.60[d] | |
| C/N | −0.25 | 0.00 | −0.34 | −0.59 | 0.03 | −0.13 | −0.36 |

[a] 10 degrees of freedom (df) for all cases except TOC and C/N which have 9 df (TOC and C/N were not measured at station 5, see Table 2). Abbreviations: TDC, total direct count; VIB, *V. parahaemolyticus* counts; COLI, fecal coliform counts; ENT, enterococci counts; SIZE, median particle size of sediments; TIDE, tidal exposure; TOC, total organic carbon; and C/N, carbon-to-nitrogen ratio.
[b] Significant correlation, $0.001 < P < 0.01$.
[c] Significant correlation, $P < 0.001$.
[d] Significant correlation, $0.01 < P < 0.05$.

TABLE 4. Multiple regression analyses of bacterial counts with the physical variables of Savin Hill Cove sediments

| Dependent variable[a] | Significant contributions of $R^2$ for independent variables[a] | | | | Total $R^2$ | Significance of equation[b] |
|---|---|---|---|---|---|---|
| | TIDE | TOC | SIZE | C/N | | |
| TDC | 0.7810[c] | | | 0.1300[d] | 0.9110 | $F_{(2,8)} = 40.94$, $P < 0.001$ |
| VIB | | | | | | |
| COLI | | 0.5454[d] | | | 0.5454 | $F_{(1,9)} = 10.80$, $P < 0.01$ |
| ENT | | 0.5345[e] | | | 0.5345 | $F_{(1,9)} = 10.34$, $P < 0.05$ |

[a] Abbreviations, see Table 3.
[b] Regression equations: TDC = [3.930 − 0.0065TIDE − 0.189(C/N)] × $10^9$; COLI = (−18.3255 + 0.719TOC) × $10^2$; ENT = (−1.168 + 0.1044TOC) × $10^2$.
[c] Significant correlation, $P < 0.001$.
[d] Significant correlation, $0.001 < P < 0.01$.
[e] Significant correlation, $0.01 < P < 0.05$.

correlation with fecal coliforms ($r = -0.73$, $P < 0.01$), distance from outfall is uncorrelated with other variables along this part of the sampling transect.

The density gradient in bacterial counts (Fig. 2) may represent a balance between the introduction of bacteria from the CSO and factors that limit their subsequent dispersal and survival in sediments. Distance from the outfall is probably a rough correlate of the rate at which sewage-borne bacteria and nutrients are introduced to surrounding sediments. Tidal exposure could measure how much the counts are reduced by limiting the period available (i.e., high tide) for colonization of sediments by bacteria from CSO effluent, resuspension and erosion of sediments during tidal cycles, and exposure of bacteria to desiccation, extreme temperature variation, and UV light. Near the CSO, high densities would be maintained by the high rate at which bacteria and nutrients are introduced and the high survival rates permitted by subtidal conditions. With increased distance from the CSO across the intertidal area, lower rates of influx of bacteria and nutrients coupled with more exposed conditions may act to reduce bacterial densities. The effect of the CSO, evinced by the presence of fecal indicators in sediment, extends at least 460 m.

Although intertidal sediments are subject to a variety of harsh conditions which might be expected to kill fecal indicator bacteria, we showed that fecal coliforms and enterococci can accumulate in sediments adjacent to a CSO, in this case up to about 0.5 km away from the point source. Shellfishing is prohibited in this study area. In Boston Harbor, 1,159 ha of productive shellfish beds are classified as restricted (Commonwealth of Massachusetts, Executive Office of Environmental Affairs Publication no. 14224-65-500-10-85 C.R., 1985). Harvesting in these beds is prohibited approximately half of the time because of high coliform counts in overlying waters. The beds are typically reclassified as restricted or prohibited on a biweekly basis. The sole criterion for reopening these affected beds is acceptable coliform counts in overlying waters. Our work shows that even when the counts from the water column are well within the Food and Drug Administration standard for approved shellfish harvest waters, which is a median fecal coliform MPN of 14 per 100 ml (7), there can be high numbers of these indicator bacteria in the sediments. Matson et al. (12) suggested monitoring fecal coliform counts in riverine sediments. Estuaries, like rivers, are subject to constant water movement and sporadic sewage inputs, which may lead to deceptively low counts of indicator bacteria in the water column. As in rivers, sediments are more likely to reflect accurately the recent history of fecal pollution in estuarine environments. Since human pathogens could be introduced into the water column by resuspension (8, 9), intertidal sediments near sewage outfalls must be considered a potentially hazardous reservoir of sewage-borne diseases.

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