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Extraction of *Clostridium perfringens* Spores from Bottom Sediment Samples

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Two extraction-separation procedures were developed and evaluated for use in conjunction with the mCP membrane filter method for the enumeration of *Clostridium perfringens* spores in bottom sediments. In the more facile of the two procedures, a distilled-water suspension of the sediment sample is pulse sonicated for 10 s and allowed to settle. Portions of the supernatant are then removed for membrane filtration. This procedure is recommended for general use. The more complicated procedure is recommended for situations in which the presence of high levels of toxic materials is suspected or in which relatively low spore densities are present in fine silts. In this procedure, sonication is followed by a distilled water wash. The centrifuged sediment is resuspended in distilled water and mixed with the components of a two-phase separation system (50% polyethylene glycol in distilled water and 25% sucrose in 3 M phosphate buffer [pH 7.1]). After equilibration of the system and low-speed centrifugation, the top phase and interphase are removed, mixed, and membrane filtered. The recoveries of *C. perfringens* spores by the two procedures, when used in conjunction with the mCP method, were comparable to each other and significantly greater than those by the British most-probable-number method. It was estimated that more than 85% of the spores were recovered by the procedures. The precision of the sonicate-and-settle-mCP procedure was markedly better than that obtained theoretically by the most-probable-number method and approached that theoretically attributable to counting an average of 85 colonies on each of two plates.

Coastal and estuarine surface waters are regularly monitored for microbial indicators of their contamination with human fecal wastes. This is done to index the potential for infectious disease among those who use these resources as sources of food and recreation. However, such measurements may understate the potential for enteric disease since the densities of certain fecal indicators in some marine sediments may exceed those in the overlying water column by as much as three orders of magnitude (16), and one of the resources in question, molluscan shellfish, is located in the benthos. Bottom sediments are subject to contamination with pathogenic microorganisms discharged in human fecal wastes in two ways, namely, from particulates which settle out of waters receiving sewage effluents and from sewage sludge barged or piped to specific disposal sites.

Both the degree to which the sediments are contaminated with sewage particulates and the consequent risk of infectious disease to users of the associated shellfish resource are generally indexed by coliform indicator systems. However, because they are more susceptible to environmental stress than are the viral pathogens (3, 12, 14; D. P. Fiedler, M.S. thesis, University of Rhode Island, Kingston, 1980; R. Vasl, M.S. thesis, Hebrew University, Jerusalem, Israel, 1978), coliforms would be expected to inconsistently reflect the health risks involved; this expectation was realized in the findings from bathing-beach epidemiological studies (7) and inferred from analyses of some shellfish-borne disease outbreaks (8, 18). Moreover, there are data showing that the adsorption of viruses to particulates further increases their survival in marine waters (15). Thus, with specific reference to sewage particulate-contaminated marine sediments, there is a need for a "longer-lived" indicator.

Clostridium perfringens is a spore-forming, obligately anaerobic bacterium that was one of the first organisms to be considered as an indicator of fecal pollution (19). It is consistently associated with human fecal wastes (1, 10, 13) and is itself a major cause of wound infections and gas gangrene. Because of the widespread distribution of its spores in both terrestrial and aquatic environments, *C. perfringens* density data must be interpreted with caution in situations in which there is significant stormwater

TABLE 1. Increase in the recovery of *C. perfringens* spores by successive extractions in the two-phase system[a]

Extraction no.	No. of *C. perfringens* spores per g of sediment[b]	% Increase[c]	Cumulative % recovery[d]
1	8,150		47.7
2	4,700	57.7	75.1
3	2,120	45.1	87.5
4	1,050	49.5	93.7

[a] Extractions were preceded by three distilled water washes, with 10 s of sonication for each wash.
[b] Sample from New York Bight station 27.
[c] Average percent increase, 50.8.
[d] Estimated *C. perfringens* density in sample, 17,100/*g* (obtained as described in text).

runoff or resuspension of contaminated bottom sediments into the water column. However, this indicator system is useful in those specific instances where (i) remote sources of pollution are to be detected, (ii) the water has come into contact with materials that are toxic to the generally used indicators, or (iii) the spores of the organism are to be used as a naturally occurring conservative tracer to examine the in situ biological decay of fecal pathogens and indicator bacteria (5, 6). In addition, the enumeration of *C. perfringens* spores in bottom sediments should prove to be useful in monitoring the deposition and movement of sewage particulates since the inactivation of the traditionally used indicators can confound the results obtained.

MATERIALS AND METHODS

Sediment samples. The sediment samples used in the development and evaluation of the *C. perfringens* spore extraction-concentration procedure were collected with Smith-McIntyre grab samplers from upper Narragansett Bay, R.I., and from the vicinity of the New York Bight sewage sludge dumpsite. The samples were held in our laboratory in the dark at 4°C and used periodically over a 1-year interval.

***C. perfringens* assays.** The extracted *C. perfringens* spores were enumerated by the mCP (4) membrane filter method. In addition, presumptively positive tubes of reinforced clostridium medium used in the British most-probable-number (MPN) method (9) were confirmed by streaking from growth-positive tubes onto plates of mCP medium. This was a substitute for the stormy fermentation of milk required for the specific enumeration of *C. perfringens* by the method. This substitution permitted a more direct comparison of the recoveries by extraction-mCP and MPN procedures.

Extraction-separation procedures. The treatment protocol initially examined for the extraction-separation of the *C. perfringens* spores from the sediment was as follows. The sample was mixed, and a weighed portion (0.05 to 10 g) was added to a sterilized 50-ml polycarbonate tube containing 5 ml of distilled water. The sample was washed with two or three 30-ml portions of distilled water. This was accomplished by a sequence of sonication (20 0.5-s pulses at 1,400 W/in^2 [ca. 215 W/cm^2] and a 2-mil amplitude in a Branson Sonifier model 350 fitted with a ⅛-in. [ca. 0.3-cm] micro tip), centrifugation (10 min at 5,860 × *g*), careful removal of the supernatant, and resuspension of the sediment in 30 ml of sterile distilled water. The further removal of toxic materials and reduction of the number of small particles which could occlude the membrane filters was attempted by using the two-phase system developed by Sacks and Alderton (20) for "cleaning up" spore preparations. The washed sediment was resuspended in 5 ml of distilled water; the suspension was mixed with the two components of the system (13 ml of 50% [wt/vol] polyethylene glycol in distilled water and 22 ml of 3 M phosphate buffer [pH 7.1]); equilibration of the system and sedimentation of the large particles were facilitated by centrifugation (acceleration in a swinging-bucket rotor to 700 × *g* and then an unbraked stop); the top (polyethylene glycol-rich) phase and interphase were removed; and appropriate portions thereof were assayed by the mCP method.

As a consequence of the results presented below, the treatment train was modified to yield two extraction-separation procedures. In the more facile of the two (sonicate-and-settle), the distilled water suspension of the sediment was sonicated for 10 s as described above, another 35 ml of sterile distilled water was added to the tube, and the contents of the tube were blended in a Vortex mixer for 10 s and then set aside for 10 min. Samples removed from the supernatant were appropriately diluted and assayed by the mCP method.

The more complicated and laborious extraction procedure (two-phase) differed from the initial treatment train in that there was a single distilled water wash, the total sonication time did not exceed 10 s, and sucrose was added to the phosphate-rich solution to a final concentration of 25% (wt/vol).

RESULTS

A portion of the sediment sample from a station near the New York Bight dumpsite was subjected to four sequential two-phase extractions as follows. After equilibration of the two-phase system containing the sonicated and washed sediment sample, the top phase, interphase, and, of necessity, a small portion of the bottom phase material were removed for assay.

TABLE 2. Effect of sonication time on recovery of *C. perfringens* spores from sediment[a]

Time (s)	No. of *C. perfringens* spores per g (wet wt) of sediment[b] ± SEM
0.0	8,100 ± 461
2.5	8,280 ± 350
5.0	8,630 ± 221
10.0	9,220 ± 234
15.0	7,470 ± 296

[a] Recovery by two-phase system.
[b] Sample from New York Bight station 23.

TABLE 3. Comparison of *C. perfringens* spore recoveries from New York Bight sediment samples by unmodified and modified extraction—separation procedures (followed by mCP membrane filtration) and by the MPN method

Source of sediment sample[a]	No. of *C. perfringens* spores recovered per g (wet wt) of sediment by:						
	Two-phase procedures[b]			Settling procedures			
	Unmodified	Sucrose[c]	% Imp[d]	Shake (unmodified)	Sonicate[e]	% Imp	MPN
NY 22	1,870	3,050	63	3,150	6,750	114	6,010
NY 24	2,060	2,930	40	1,880	2,750	46	250
NY 26	449	515	15	278	594	114	95
NY 27	7,110	16,200	128	1,440	15,500	976	1,570
NY 40	724	923	27	940	958	19	160
NY 45	19,800	25,500	29	15,400	21,300	38	4,310
GM	2,370	3,480		1,800	3,890		733

[a] NY, New York Bight station; GM, geometric mean.
[b] One two-phase extraction.
[c] Sucrose added to a final concentration of 25% in the phosphate-rich solution.
[d] Percent improvement of modified over unmodified procedure.
[e] Includes two distilled water washes, with 10 s of sonication per wash.

They were replaced with the comparable portions from a freshly equilibrated mixture of the two solutions. The contents of the tube then were blended in a Vortex mixer for 5 min, and the resulting mixture was equilibrated by centrifugation. This treatment sequence was repeated until the four extractions were completed. The numbers of *C. perfringens* spores recovered during each extraction are shown in Table 1. It can be seen that the number recovered during a given extraction was, on the average, 50% of that obtained during the previous one. The estimated spore density in the sample, obtained as the asymptote from successive projected 50% increases in recovery, was 17,100/g. This value was used to calculate the cumulative percent recovery for each extraction shown in the table. At least three extractions were necessary to obtain a reasonable recovery (87%) of the spores in the sample. In two further experiments, it was observed that the centrifugation used to equilibrate this system during two-phase extraction increased the recovery of *C. perfringens* by about 24% and that the spore recovery increased with increased sonication up to but not exceeding 10 s (Table 2).

The effort required for three successive two-phase extractions was considered a deterrent to routine use of the procedure. The first approach toward simplifying the procedure was derived from our reasoning that a significant improvement in the recovery efficiency from a single extraction might be achieved by increasing the density of the phosphate layer without diminishing its highly hydrophilic character; some preliminary experiments (data not shown) suggested that this could be accomplished by the addition of sucrose to a final concentration of 25% in the phosphate-rich solution. The second approach was the elimination of the two-phase extraction step altogether. It was, in effect, a modification of a simple shake-and-settle treatment (5 min of blending in a Vortex mixer followed by 10 min of settling) in which shaking was replaced by the combination of sonication and two distilled water washes.

The two-phase procedure, its two modifications, the shake-and-settle-treatment, and the British MPN method were compared for the recovery of *C. perfringens* spores from sediment samples collected at six New York Bight stations. The results from this experiment are given in Table 3. Analysis of the variance of the $\log_{10}$-transformed data indicated that (i) the recovery by the two-phase procedure was significantly improved ($P = 0.012$) by the addition of sucrose to the phosphate-rich solution; (ii) the modification of the shake-and-settle procedure by the combination of sonication and washing (sonicate and settle in Table 3) also improved the spore recovery, by 19 to 976%, and the increases approached significance ($P = 0.07$); and (iii) the recoveries by the two modified methods were not significantly different from each other but were significantly greater than those by the MPN method ($P < 0.001$).

Since the improved recoveries by the sonicate-and-settle as compared with the shake-and-settle treatment (Table 3) could be ascribed to sonication or the two prewashes or both, the possibility that one of these two steps could be eliminated was pursued in some further experiments. In the first, it was observed that the substitution of 10 s of sonication for the 5 min of blending in a Vortex mixer in the shake-and-settle procedure increased the *C. perfringens*

TABLE 4. Requirement for a prewash in the sonicate-and-settle extraction procedure

Source of sample[a]	No. of C. perfringens spores recovered[b] per g with:		
	No wash	Distilled water	
		1 wash	2 washes
NY 26	745	739	667
NY 27	14,200	11,900	13,400
NY 45	51,500	42,500	43,500

[a] NY, New York Bight station.
[b] Recoveries not significantly different.

recovery by 46%. This confirmed the results in Table 2 on the need for the more vigorous disaggregation treatment, i.e., sonication. Furthermore, the *C. perfringens* recoveries from three sediment samples were not improved by the preliminary distilled water washes (Table 4). Essentially, the same findings were obtained when the prewash step in the two-phase procedure was examined, and the substitution of phosphate buffer (pH 7.1), citrate buffer (pH 5.0), or glycine buffer (pH 2.5) for the distilled water did not improve the recovery of *C. perfringens* over that in the control (no wash) (data not shown). These findings indicated that the improvements noted above and shown in Table 3 were due to the sonication treatment, presumably by the disaggregation of multispore-containing particles, and not to the prewash. Therefore, sonication, but not the prewash, was retained as an essential step in both the two-phase and the sonicate-and-settle extraction-separation procedures. The comparability of the *C. perfringens* spore recoveries by the two modified procedures can be seen from the results shown in Table 5.

The results obtained thus far showed that the *C. perfringens* spore recoveries from sediment samples by the two simplified extraction procedures were comparable to each other and significantly higher than those obtained with the MPN method. However, they did not show the *C. perfringens* recovery efficiencies (accuracy) of the two procedures in a more absolute sense. The accuracy of the two-phase procedure was estimated to be in excess of 85% by the following calculation: $47.7 \times (9{,}220/7{,}740) \times (3{,}480/2{,}370) = 86.4$. The recovery by a single two-phase extraction preceded by three washes (30 s of sonication) was 47.7% (Table 1). The reduction of the 30-s sonication period to 10 s should have increased the recovery at least by a factor of 9,220/7,470 (Table 2), and the addition of sucrose to the phosphate-rich solution increased the recovery, on the average, by a factor of 3,480/2,370 (Table 3). Since the two procedures produced comparable recoveries (Table 5), the extrapolated estimate for the two-phase procedure was attributed to the sonicate-and-settle method as well.

The estimated recovery efficiencies could not be confirmed by seeding known numbers of *C. perfringens* spores into relatively spore-free sediments. Doing so would have circumvented a major requirement of the extraction-separation procedures, namely, the disaggregation of multiple numbers of spores contained in the relatively large sewage particles which settle into the bottom sediments, and the demonstrated need for sonication was consistent with this requirement. An alternative approach was to use the MPN procedure to estimate the number of spores retained in the sediment after the extraction-separation procedures. However, this approach also had a shortcoming; the number of *C. perfringens* spores remaining in the extracted sediment would be underestimated to the extent that the sonication treatment did not disaggregate the spores completely. The estimated recovery efficiency of the sonicate-and-settle method was in excess of 90% (Table 6). From the results shown in Table 5, we inferred that this was also true of the modified two-phase procedure.

The comparison of the *C. perfringens* spore recoveries obtained at two different times from the same sample (compare the data shown in Tables 3 and 5) led us to question the precision of the extraction procedures. However, since the sediment samples were scraped from the surface of Smith-McIntyre grabs and not thoroughly mixed upon collection, an alternate explanation was variability in the spore content of the samples themselves. This issue was resolved by the analysis of the data acquired from duplicate extraction-assays by the sonicate-and-settle procedure of 87 sediment samples subsequently collected from the New York Bight. In this instance, however, each sample was thoroughly mixed before the paired extractions-assays were performed. The *C. perfringens* spore densities in the samples ranged from 2.5×10^1 to 8.2×10^4.

TABLE 5. Comparative recoveries of *C. perfringens* spores by the sonicate-and-settle procedure and the two-phase system[a]

New York Bight sample station no.	No. of spores recovered by:	
	Sonicate-and-settle method	Two-phase system
22	6,730	5,400
24	3,470	3,550
26	970	900
34	134	150
40	480	450
43	13,900	14,200
45	46,100	46,700

[a] No wash with either method; 10 s of sonication.

TABLE 6. Loss of *C. perfringens* spores to the bottom material in the sonicate-and-settle method as estimated by the MPN procedure

Sample no.[a]	No. of *C. perfringens* spores per ml in top 25 ml	No. of *C. perfringens* spores per ml in bottom 15 ml	Avg no. of *C. perfringens* spores per ml (*A*)	Vol (ml) remaining for MPN (*B*)	No. of *C. perfringens* spores (*C*)[b]	*C. perfringens* MPN in vol remaining (*D*)	No. of *C. perfringens* spores not counted (*E*)[c]	Total no. of *C. perfringens* spores (*F*)[d]	% Loss[e]
23									
Rep 1	343	316	330	1.8	594	1,300	706	14,500	4.9
Rep 2	295	337	316	1.5	474	1,700	1,230	14,300	8.6
27									
Rep 1	657	660	659	0.4	264	500	236	26,900	0.9
Rep 2	643	639	641	0.5	321	800	479	26,500	1.8

[a] Rep, Replicate.
[b] $C = A \times B$.
[c] $E = D - C$.
[d] $F = 40A + D$.
[e] Percent loss = $(E/F) \times 100$.

Coefficients of variation (CVs) were obtained for each pair of values. The $\log_{10}$ of the CVs, but not the CVs themselves, were normally distributed (Fig. 1 [every fifth point and the last three are shown]). The median CV was 8.4%. This value approaches the theoretical CV (7.4%) for counting two plates at the upper counting limit (85 colonies per membrane) of the mCP method (4) and is much less than that for two five-tube, three-dilution MPN tests (about 50%) (21).

DISCUSSION

Either of the two extraction-separation methods, when used in conjunction with the membrane filter (mCP) assay, provides more accurate and precise estimates of *C. perfringens* spore densities in bottom sediments than does the British MPN method (9). The better precision derives from counting colonies instead of positive tubes. The greater accuracy (significantly better recoveries) is due to disaggregation of particles containing two or more of the *C. perfringens* spores and may also derive from better activation of the spores during sonication. In addition, there may have been soluble or particle-associated inhibitors of the growth of *C. perfringens* in the sediment samples; and the latter were reduced during settling, and the former were reduced during membrane filtration. MPN assays for other microbial indicators in sludge or sludge-contaminated sediments may be similarly affected. In some preliminary studies, we found that, with some of the same sediment samples collected near the New York Bight sludge dumpsite, the *Escherichia coli* and enterococcus recoveries as obtained by the mTEC (11) and mE (17) methods, respectively, were markedly greater than those by the MPN methods for fecal coliforms and fecal streptococci (2).

Because of its relative simplicity, the sonicate-and-settle procedure is recommended for general use. The data clearly show that blending in a Vortex mixer cannot be used as an alternative to sonication. Other equally rigorous but nondestructive disaggregation measures may obviate the need for a Sonifier. Our own attempts to employ the combination of blending in a Vortex mixer and surfactants (data not shown) were largely unsuccessful, presumably because of the fecal nature of the sewage particles. The two-phase procedure may be useful in those instances where low spore densities are anticipated in sediments which are composed of very fine silts or contain excessive amounts of substances which inhibit the growth of the organism. To this end a single distilled water wash was retained in the procedure, as described above.

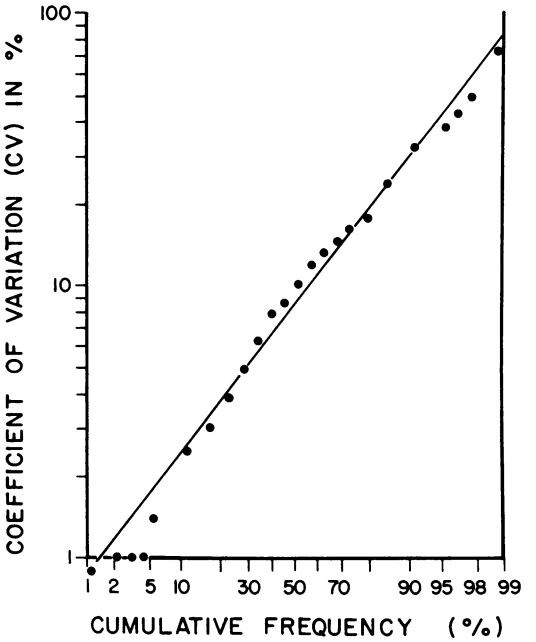

FIG. 1. The cumulative frequency distribution of the CVs for *C. perfringens* spore recoveries from paired sonicate-settle-extraction-mCP assays of 87 sediment samples. Points are shown for every fifth value, except for the first and last five.

The findings from the application of the methodology developed are being prepared for publication. The density of *C. perfringens* spores in bottom sediments was found to be a very sensitive indicator of the deposition and movement of sewage particulates. Moreover, *C. perfringens* spore densities, in and of themselves and when compared with those of other fecal indicators, provided information on the nature and proximity of fecal inputs into aquatic environments.

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