

Membrane Filter Enumeration Method for *Clostridium perfringens*

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A membrane filter procedure has been developed for the rapid quantitation of *C. perfringens* in the aquatic environment. Background growth is inhibited by the use of D-cycloserine, polymyxin B sulfate, and incubation at 45°C. Differential characteristics include the fermentation of sucrose, production of acid phosphatase, and the absence of β -D-glucosidase activity. The medium is prepared as follows (in grams per 100 ml of distilled water): tryptose, 3.0; yeast extract, 2.0; sucrose, 0.5; L-cysteine, 0.1; MgSO₄·7H₂O, 0.01; bromocresol purple, 0.004; and agar, 1.5. The ingredients are dissolved, and the pH is adjusted to 7.6. After autoclaving at 121°C for 15 min, the medium is allowed to cool at 50°C, and the following are added per 100 ml: D-cycloserine, 40 mg; polymyxin B sulfate, 2.5 mg; indoxyl- β -D-glucoside, 60 mg; 2.0 ml of a filter-sterilized 0.5% phenolphthalein diphosphate solution; and 0.2 ml of a filter-sterilized 4.5% FeCl₃·6H₂O solution. Enumeration of *C. perfringens* in a water sample is completed within 18 to 24 h. The verification of typical colonies was 93%. The average recovery from peptone-water spore suspensions of five strains was 79%, and that from filter-sterilized seawater suspensions was 90%. The precision of the method was approximately equal to that expected from random error alone. Confirmed recoveries of *C. perfringens* from water and sewage samples generally were greater than those by the Bonde pour tube method.

The use of *Clostridium perfringens* as a water quality indicator has been a subject of considerable controversy (3, 21, 23, 26) since 1899 when Klein and Houston (15) first suggested that this organism could be used to detect fecal pollution. It is rarely used as such in the United States, for the reasons stated by Levine (17) and summarized by Prescott et al. (21). However, a number of European workers (23, 26) continued to recommend *C. perfringens* as a valuable supplement to other water quality tests, particularly in certain specific situations. Included are (i) the examination of chlorinated waters and untreated water containing industrial wastes lethal to non-sporeforming bacteria, samples collected more than 12 h before assay (3), and sewage sludges (3, 23), and (ii) situations in which the detection of remote as well as recent pollution is desirable, soil run-off and resuspension of bottom sediments are not significant, or the survival properties of the water quality indicator are at a premium (3, 5).

At least part of the controversy over the value of the *C. perfringens* indicator system may have been due to the measurement of a heterogeneous group of organisms, "sulfite-reducing, spore-

forming anaerobes" (26) on one hand or the use of a rather imprecise method (most probable number; MPN) on the other. The development of facile methodology which would provide accurate, precise, and specific estimates of *C. perfringens* spore and vegetative cell densities from relatively large samples of environmental water is needed to resolve some of these questions. The report to follow will deal with the development of such methodology.

Currently, there are several methods available for the quantitation of *C. perfringens* from the aquatic environment. Included among these are a most probable number procedure (7), several pour plates methods (1, 11, 12, 18, 22), a membrane filter method (draft report of SC4/WG5 meeting on sulfite-reducing sporeforming anaerobes [clostridial], International Standards Organization, Berlin, 1975), and the Bonde pour tube method (BPT; 3). All of these methods use sulfite reduction as the differential characteristic in the primary medium, and some use stormy fermentation of milk for specific identification. However, stormy fermentation of milk is not a suitable test for colony-counting procedures; and sulfite reduction alone is not specific enough for

the identification of *C. perfringens*. Furthermore, all these methods suffer from one or more of the following deficiencies: (i) if *C. perfringens* rather than sulfite-reducing, sporeforming anaerobes are to be enumerated, colonies or tubes have to be subcultured for identification, and this is time consuming and often difficult; (ii) most-probable-number methodology with its attendant imprecision is used; (iii) large volumes of water cannot be examined; and (iv) colonial morphology cannot be used in identification. Therefore, the development rationale for the method to be presented was the inclusion of a number of compatible, differential tests in a selective medium to be used in a membrane filter procedure. Thereby, large quantities of water could be examined, colony counts would be obtained, and *C. perfringens* specifically would be enumerated without the need to routinely pick colonies for identification.

MATERIALS AND METHODS

Cultures. Five *C. perfringens* strains were used in the development of the mCP method. Two of these (C-1861 and FD-2) were provided by V. Dowel (Center for Disease Control, Atlanta, Ga.), and the others (strain no. 2, 9, and 15) were isolated from Narragansett Bay, R.I. Stock spore suspensions of all five strains were obtained by the procedure described by Ellner (9) and that described by Duncan and Strong (6). After the final wash, the spores were concentrated by centrifugation and resuspended in Sorensen phosphate buffer (pH 7.2). The suspensions were dispensed in 5.0-ml amounts into sterile test tubes and stored at 6°C.

Test suspensions were prepared immediately before use by serial dilution of the spore stocks in 0.1% peptone-water or filter-sterilized seawater.

Recovery medium. The recovery medium developed (mCP) was prepared by adding the following ingredients (in grams per 90 ml) to distilled water: tryptose, 3.0; yeast extract, 2.0; sucrose, 0.5; L-cysteine hydrochloride, 0.1; MgSO₄·7H₂O, 0.01; bromocresol purple, 0.004; and agar, 1.5. The ingredients were dissolved, and the pH was adjusted to 7.6. After autoclaving at 121°C for 15 min, the medium was allowed to cool to 50°C and the following ingredients were added: 40 mg of D-cycloserine (Sigma Chemical Co.) and 2.5 mg of polymyxin-B sulfate (Sigma) as the dry ingredients; indoxyl β-D-glucoside (IBDG; Reliable Chemical Co.), 60 mg dissolved in 8.0 ml of sterile distilled water; 2.0 ml of a filter-sterilized, 0.5% solution of phenolphthalein diphosphate (Sigma); and 0.2 ml of a filter-sterilized 4.5% solution of FeCl₃·6H₂O. Once it had cooled to 50°C, the medium was dispensed in 5-ml quantities into sterile petri dishes (50 by 12 mm). The poured plates were stored in an anaerobic jar (Baltimore Biological Laboratory [BBL] GasPak anaerobic unit) until use.

The development of the membrane filter procedure for *C. perfringens* started from the reported ability of *C. perfringens* spores to be quantitatively recovered on a tryptose—yeast extract medium (22) and the

ability of the organism to grow anaerobically at temperatures in excess of 40°C (3, 4). The pH indicator, bromocresol purple, was added to identify colonies that were able to ferment sucrose. Magnesium and iron salts were added to the medium in low concentrations, since Fuchs and Bonde (10) reported that their presence enhances the growth of *C. perfringens*. Results in our laboratory concurred with their findings.

Sucrose fermentation. From the examination of the sugar fermentation reactions for the various species of *Clostridium* (13), it was concluded that the fermentation of sucrose differentiated *C. perfringens* from the greatest number of other species in the genus.

β-D-Glucosidase activity. Cellobiose (glucose β-D-glucoside) is not fermented by any strains of *C. perfringens* (13). When the requirement not to ferment cellobiose is added to the one for sucrose fermentation, only four species in addition to *C. perfringens* would be scored as positive (13). For obvious reasons, both sugars could not be incorporated into the primary medium. However, Dufour (personal communication) obviated a similar difficulty in a method for coliforms by using indoxyl β-D-glucoside instead of cellobiose. Colonies of cells which hydrolyze the glucoside turn blue from the production of indigo blue.

Acid phosphatase test. Ueno et al. (25) and Porschen and Spaulding (20) reported that the ability of *C. perfringens* to produce acid phosphatase differentiated it from a number of other species of *Clostridium*. Blazevic and Ederer (2) described a test for phosphatase activity using phenolphthalein diphosphate as a substrate. When phenolphthalein diphosphate is incorporated into the base medium, the phosphate is cleaved from the substrate molecule by the action of acid phosphatase, and typical colonies of *C. perfringens* turn a dark pink (Phos⁺) after exposure to an opened flask of ammonium hydroxide. The effectiveness of this differential procedure could not be predicted, since Ueno et al. (24) examined only 14 of the 60 or so described species. Therefore, a fourth biochemical characteristic, gelatinase activity, was used initially to presumptively identify *C. perfringens*.

In situ gelatin test. The hydrolysis of gelatin (gel) is a major characteristic in the differentiation of *C. perfringens* from other clostridia (4, 13). To utilize this characteristic and still avoid the need for subculturing colonies for identification, an in situ test for gelatinase activity was developed after the reported success with in situ tests by Watkins et al. (25). After the performance of the acid phosphatase test, the mCP was transferred with sterile forceps from the mCP medium to a plate of the gelatin medium. After 1 to 2 h of incubation at 37°C, the filter was removed from the in situ medium, which was then flooded with 1 to 2 ml of Frazier's reagent. Colonies on the filter corresponding to hydrolysis in the medium were scored as positive. Suc⁺, Cello⁻, Phos⁺, Gel⁺ colonies were counted as "presumptive" *C. perfringens*.

The ingredients for the gelatin medium used in the in situ test were as follows (in grams per 100 ml): tryptone, 1.5; yeast extract, 1.0; gelatin, 1.5; and agar, 1.5. The ingredients were dissolved, autoclaved at 121°C for 15 min, and dispensed in 3.0-ml amounts of sterile petri dishes (50 by 12 mm). Frazier's reagent consisted of 5 g of HgCl₂, 20 ml of concentrated HCl, and 100 ml of distilled water.

Confirmation of colonies. When it was necessary to pick colonies for verification, all plates were examined under flowing nitrogen. The short exposure of the colonies to the ammonia vapors did not affect viability. Colonies presumptively identified as *C. perfringens* by the mCP method, as well as those not identified as such, were picked into tubes of prereduced peptone—yeast extract—glucose (PYG) broth and incubated at 37°C for 18 h. The isolates were then submitted to a series of biochemical tests (4, 13) that allowed either their confirmation or their rejection as *C. perfringens*. The colonies were confirmed by the following criteria: gram-positive rod; obligatory anaerobic; nonmotile; fermentation of lactose, mannose, and sucrose with the production of gas; failure to ferment cellobiose, mannitol, and salicin; stormy fermentation of milk; production of lecithinase, gelatinase, and acid phosphatase; reduction of sulfite to H₂S and of nitrate to nitrite.

Control medium. The control medium used in the pure-culture recovery experiments was the basal medium of the sulfite—polymyxin—sulfadiazine agar described by Angelotti et al. (1). This medium was reported to recover *C. perfringens* in numbers equal to other nonselective media (12). Results obtained in our laboratory concurred with this finding. The resultant medium, tryptose—yeast extract—iron citrate agar (TYI), was prepared by dissolving the following ingredients (grams per 100 ml) in distilled water, adjusting the pH to 7.0, and autoclaving at 121°C for 15 min: tryptone, 1.5; yeast extract, 1.0; agar, 1.5; and iron citrate, 0.05.

Membrane filtration. Appropriate volumes of the test suspensions and water samples used in evaluating the mCP method were passed through membrane filters (HCWG; Millipore Corp.). Volumes less than 20 ml were brought to approximately that amount with phosphate-buffered saline (16) before filtration.

Samples. All samples were collected in sterile containers and assayed within 8 h of collection.

RESULTS

Initially, experiments were conducted to determine the peptone source for the medium. Since one of the more commonly used plating media for *C. perfringens* contains tryptone (1) and a second tryptose (23) these two peptone sources at a concentration of 1.5% (wt/vol) were compared for the recovery of *C. perfringens* spores in a base which contained 1.0% egg yolk, 0.5% sucrose, 0.004% bromocresol purple, 1.5% agar, and 1.0% yeast extract as a source of vitamins and growth factors. Tryptose was selected, since the recoveries of spores from strains 9 and FD-2 were consistently, although not significantly, higher with this peptone source. Nonetheless, the *C. perfringens* recoveries on membranes applied to the tryptose-containing medium were lower by about 20% than those with the control TYI agar pour plates. It was suspected that the filters themselves were preventing the nutrients from reaching the spores in sufficient concentrations. Therefore, the effects

of increasing the concentration of tryptose and yeast extract and the addition of polypeptone were examined in a second series of experiments. The maximum recoveries were obtained with concentrations of tryptose and yeast extract of 3 and 2%, respectively, and the addition of 0.5% polypeptone to this formulation did not further increase the recovery of the spores. The average recovery relative to TYI pour plates for the three strains examined was over 90%.

Fuchs and Bonde (10) reported that *C. perfringens* failed to grow in a medium completely lacking iron and that growth was enhanced by the addition of increasing quantities of ferrous ion to the medium until a maximum concentration of 0.6 mg/liter was attained. When small amounts of iron citrate were added to the medium under development, there was an appreciable increase in the recovery of strain 9 and a less pronounced one with strain FD-2. Therefore, several different iron compounds at various concentrations were examined for their influence on spore recovery. Maximum recovery of *C. perfringens* was attained when the base medium was supplemented with 9 mg of ferric chloride per 100 ml of medium. This concentration was reached by adding 0.2 ml of a filter-sterilized, 4.5% solution of ferric chloride.

Three antibiotics, neomycin, polymyxin B sulfate, and D-cycloserine, were examined for their abilities to inhibit background growth while permitting the quantitative recovery of *C. perfringens* on the medium. The maximum concentrations of D-cycloserine and polymyxin B sulfate which did not significantly reduce the recovery of *C. perfringens* were 40 and 2.5 mg per 100 ml of medium, respectively. The combination of these two antibiotics in the concentrations given resulted in the greatest reduction of background colonies without affecting the recovery of *C. perfringens* from natural samples.

The optimum incubation temperature was determined from the recovery of *C. perfringens* from spore suspensions and from water samples collected at locations in Rhode Island. In *Bergey's Manual* (4), it was reported that the optimum temperature for growth of *C. perfringens* is 45°C, whereas Fuchs and Bonde (10) reported it to be between 37 and 44°C. Bonde (3) used an incubation temperature of 48°C with his pour tube method. Three incubation temperatures were examined in the present study (41, 45, and 48°C). The mean recovery for each assay was obtained from three replicate filters. Those from the spore suspensions were best when the mCP plates were incubated at 41°C. With natural samples, the greatest recoveries were obtained at 45°C, although the differences among the three incubation temperatures were not signifi-

cant. Because of this and because 44 to 45°C incubation temperatures are used to assay other indicators, 45°C was chosen.

The incubation period at 45°C was also examined during the development of the method. In groups of three, nine replicate filters for each test strain were incubated at 45°C in anaerobic jars. Colony counts were obtained from the separate sets of filters at 18, 24, and 48 h. The mean recovery of *C. perfringens* spores was determined for each time period. Incubation periods in excess of 24 h did not significantly increase the recovery of *C. perfringens* from spore suspensions of strains 9, FD-2, and 2. In fact, counting was so difficult at 48 h because of overlapping colonies consequent to the increase in colony size that decreased recoveries were obtained. The results indicate that 18 to 24 h of incubation at 45°C is adequate for the recovery of *C. perfringens*.

The differential test for lecithinase production, as seen by the precipitation of the egg yolk included in the medium, was unsatisfactory. The zones of precipitation tended to coalesce, especially when the filter was crowded, making enumeration difficult. Therefore, the test for acid phosphatase activity as described above was examined as a possible substitute. Preliminary data suggested that the acid phosphatase test was specific enough for *C. perfringens* to eliminate the lecithinase test; and the egg yolk was removed from the medium once it was shown that this did not affect the recovery of the *C. perfringens* spores. Nevertheless, the specificity of the procedure did not meet the established goal, since confirmation of presumptively positive colonies was less than 90%. Up to this point in time, a presumptively positive colony was one that was positive for sucrose (Su⁺), acid phosphatase (Phos⁺) and gelatinase (Gel⁺). Our colony verification studies confirmed that, with infrequent exceptions, *C. perfringens* does not ferment cellobiose (Cello⁻). That is, less than 1% of the isolates which verified by the remaining characteristics were cellobiose positive. In addition, Su⁺, Phos⁺, and Gel⁺ colonies which did not verify as *C. perfringens* were frequently cellobiose positive as well. Thus, if the inability to ferment cellobiose were added to the other tests used in the mCP method, very rarely would colonies other than *C. perfringens* be scored as positive. For the reasons stated earlier, IBDG rather than cellobiose was incorporated into the medium. Before the incorporation of IBDG, the overall false-positive rate was 10.4%, a number slightly higher than the established limit. About 80% of the false-positive colonies fermented cellobiose. After this modification, the false-positive

rate was reduced to 5.7%, a value that was well within the preestablished goal of a 90% confirmation rate.

The final modification of the original method, the elimination of the in situ gelatinase test, simplified the procedure even further. Due to the recognized importance of gelatin liquefaction in the identification of *C. perfringens* (4, 13), gelatinase activity was included as one of the differential tests. It was performed as an in situ test because of the incompatibility of sucrose fermentation with proteolytic activity and coalescence of the zones of hydrolysis in the primary medium. The marked specificity after the addition of the IBDG test suggested the possibility of this further modification. The in situ gelatinase test was eliminated when it was found that more than 98% of over 3,000 Su⁺, Cello⁻, Phos⁺ colonies examined were also Gel⁺. The colonies came from samples of seawater, fresh water, and sewage collected from locations in six states.

The mCP procedure is outlined in Fig. 1. After incubation at 45°C, typical *C. perfringens* colonies were 1.0 to 3.0 mm in diameter, convex with entire edges, somewhat opaque, slightly butyrous in consistency, and a pale, yellow color. Upon exposure to ammonia vapors, they turned a pink to red (not purple) color.

The accuracy of the mCP method was determined by comparing the recoveries of *C. perfringens* from spore suspensions in peptone-water and sterilized seawater to those obtained with

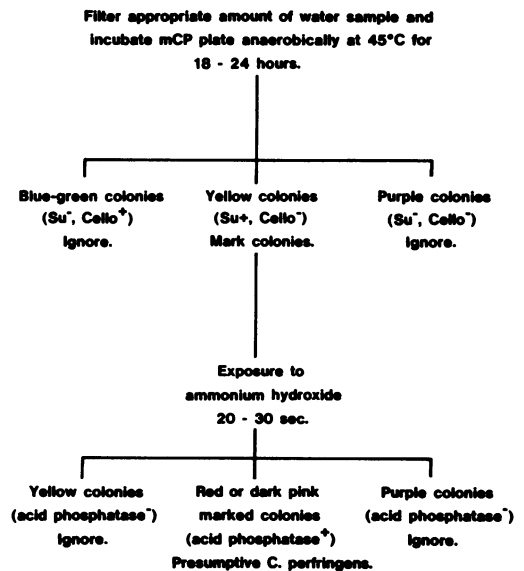


FIG. 1. Flow scheme for the conduct of the mCP procedure.

control, TYI agar pour plates. Several such trials were conducted with each strain of *C. perfringens* used in the study. The average recovery for each trial was determined from three replicate TYI pour plates and mCP filters. The relative recoveries of the five *C. perfringens* strains are shown in Table 1. The overall relative recovery of the *C. perfringens* strains from peptone—water spore suspensions was 79%, whereas that from the sterilized seawater suspensions was 90%. In most of the trials, *C. perfringens* assays using the BPT method (3) were also performed. The relative recoveries by this procedure are included in Table 1. They were considerably lower than those obtained by the mCP method.

The specificity of the mCP procedure was determined by examining several different types of water samples from various locations. Typical colonies presumptively identified as *C. perfringens* and colonies not designated as this organism were verified by the biochemical tests noted earlier. The results for more than 500 colonies are shown in Table 2. Of those colonies presumptively identified as *C. perfringens*, 93% were verified as such, and only 2.0% of the presumptively negative colonies were identified as *C. perfringens*.

The combination of anaerobic incubation at 45°C and the inhibitors included in the medium reduced the density of heterotrophic organisms other than *C. perfringens* by at least three orders of magnitude. Nevertheless, with some surface water samples, particularly those requiring the examination of 100 ml or more or those collected near a source of pollution, there were some "background" colonies. Although this complicated the isolation of pure cultures for verification, the small size of the colonies did not prevent reliable *C. perfringens* estimates from being

obtained in the proscribed counting range.

The selectivity of the mCP method for *C. perfringens* spores could also be increased by heating the sample to destroy vegetative cells, including those of *C. perfringens*. However, as Bonde (3) notes, this distinction may be a useful parameter. Some preliminary experiments with spore suspensions which had been stored in the refrigerator for several months indicated that the temperature-time regimen which he used, 80°C for 5 min, markedly reduced the recovery of the spores by the mCP method. Therefore, the survival of *C. perfringens* spores at various temperatures was examined to arrive at an acceptable treatment. Spore suspensions produced

TABLE 1. Relative recoveries from *C. perfringens* spore suspensions by the mCP and BPT methods

Strain	Recovery from spore suspension in: ^a			
	0.1% Peptone—water ^b		Filter-sterilized seawater ^c	
	mCP	BPT	mCP	BPT
9	80.9 (8)	13.8 (5)	86.3 (2)	54.1 (1)
C-1861	78.3 (7)	41.1 (4)	82.0 (2)	44.1 (2)
2	81.1 (5)	0.2 (3)	98.3 (2)	0.01 (2)
15	66.0 (6)	1.7 (5)	93.7 (2)	9.7 (2)
FD-2	87.7 (6)	26.6 (5)	90.2 (2)	47.5 (2)
Average	78.8	16.7	90.1	31.1

^a Values indicate average per cent relative to recovery with the control medium (TYI agar). Values in parentheses indicate number of trials. Three replicate filters or plates were assayed for each trial.

^b Spore suspensions were diluted in 0.1% peptone—water and assayed immediately.

^c Spore suspensions were diluted in filter-sterilized seawater, assayed immediately, held at 5°C for 24 h, and assayed a second time. There was no statistical difference between the recoveries; hence, the two assays were averaged.

TABLE 2. Verification of colonies recovered by the mCP procedure

Sample source		Typical colonies		Other colonies		
State	No. of locations	Type ^a	No. examined	No. false positive ^b	No. examined	No. false negative ^c
Rhode Island	4	SW	170	17	83	4
Connecticut	1	SW	8	1	8	0
Massachusetts	1	SW	29	0	1	0
Louisiana	1	B	22	0	17	0
Rhode Island	1	FS	6	0	12	0
Maryland	1	FS	10	2	17	0
Rhode Island	1	Sew	61	1	62	0
Total			306	21	200	4.0
% False				6.9		2.0

^a SW, Seawater; FS, fecal sample; FS, fresh water; B, brackish water; and Sew, sewage.

^b Identified as other than *C. perfringens*.

^c Identified as *C. perfringens*.

by two different methods were used at each of the temperatures examined between 60 and 80°C. The recoveries of the spore suspensions were determined from 3 TYI agar pour plates. It can be seen (Fig. 2A) that the method by which the spore suspensions were produced did influence their heat stability, that heating the suspensions at 80°C even for 5 min markedly reduced the recovery of the spores, that a first-order death curve with a reasonably shallow slope was obtained at 60°C, and that the recovery was reduced by only 15% when the suspensions were heated at 60°C for 15 min.

The effectiveness of the 60°C, 15-min heating regimen in reducing the number of background organisms on mCP medium was examined by comparing the numbers of colonies which developed from 22 heated, as opposed to unheated, samples of seawater, fresh water, and sewage collected from 13 locations. Heating the samples at 60°C for 15 min further reduced the background colonies on mCP by about 45%.

The survival of vegetative cells was examined to insure that heating samples at 60°C for 15 min would destroy most of the vegetative cells of *C. perfringens*, leaving only the spore population. Two strains of *C. perfringens* (C-1861 and 9) were grown in PYG broth for 18 h at 37°C. Slide preparations of each culture were examined for the presence of spores. None were observed in either culture after microscopic examination of more than 60 fields of vision, each of which contained between 300 and 600 cells.

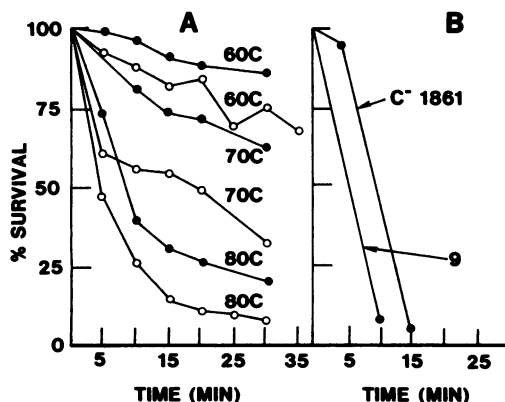


FIG. 2. Survival of *C. perfringens* consequent to heating spore and vegetative cell suspensions for various periods of time. (A) Buffered-saline suspensions of spores, prepared by the methods of Ellner (○) and of Duncan and Strong (●), were heated at 60–80°C and assayed in triplicate on TYI agar pour plates. (B) Buffered-saline suspension of 18-h PGY cultures of strains C-1861 and 9 were heated for various periods of time at 60°C and assayed in triplicate on TYI agar spread plates.

The cultures were then diluted in phosphate-buffered saline, and the survival of the cells held at 60°C for various periods of time was examined. TYI agar spread plates were used as a control. All work was performed in an anaerobic tent to eliminate any killing effect of O₂ on the vegetative cells. It can be seen (Fig. 2B) that over 90% of the vegetative cells were destroyed after 15 min at 60°C. Thus, the mCP assay with the heated suspension provides an estimate of the *C. perfringens* spores, and the differential recovery from unheated and heated samples provides an estimate of the vegetative cells. However, an unknown factor was the extent to which environmentally "stressed" vegetative cell populations of *C. perfringens* were reduced by the procedure even with unheated samples. This factor may have been agitation and oxygenation during dilution and filtration, simultaneous exposure of the cells to nutrients and oxygen before the establishment of anaerobic conditions in the GasPak jar, and/or the inhibitors in the medium.

The first experiments were designed to determine the efficiency with which the mCP method recovered vegetative cells of *C. perfringens*. Vegetative cell cultures of strains C-1861 and 9 were obtained by inoculating PYG broth and incubating the cultures for 18 h at 37°C. As before, microscopic examination of the cultures did not reveal the presence of any spores. Serial dilutions of the cultures in phosphate buffer were made inside an anaerobic tent, and the cell density for each strain was determined from three TYI agar pour plates. The mCP counts were determined from three replicate membranes by normal (aerobic) filtration. Vegetative cells of strain 9 were recovered quantitatively by the mCP procedure, as seen from the comparison of the mCP and TYI counts. Such was not the case with strain C-1861, in which the recoveries by mCP were about 30% of those by TYI.

The ability of the mCP method to recover vegetative cells of *C. perfringens* from fresh- and marine-water samples was examined in two subsequent series of experiments using the "sensitive" strain, C-1861. In the first, an 18-h vegetative cell culture was diluted and held in an anaerobic tent using anaerobically tempered, filter-sterilized fresh and marine water. The resultant suspensions were stored at 35°C and assayed after 0, 2, and 24 h. The average recovery for each assay was determined from duplicate control plates and mCP filters. Each assay was performed as follows. While in the tent, the samples were shaken and diluted in tempered phosphate buffer; portions were spread on TYI agar plates and assayed by the mCP method. The control and mCP plates were placed in a BBL GasPak jar, which was sealed, removed

from the tent, and incubated at 45°C. The appropriate dilution tubes of the suspensions were removed from the tent and shaken to oxygenate them; portions were spread on TYI agar plates and assayed by the mCP method. The second set of control and mCP plates was then placed in an anaerobic jar and incubated at 45°C. The results of this experiment are shown in Table 3. It can be seen from these data that the type of water in which the cells were suspended markedly influenced their survival. With the seawater suspension, the recoveries at zero time by the control and mCP procedures were approximately the same, whether the assay (not the incubation) was performed aerobically or anaerobically. However, after 2 h of storage, the recoveries by the mCP method were significantly lower than those with the control spread plates, particularly with the aerobic assay. No vegetative cells were recovered after 24 h by either the control or mCP methods at the examined dilutions.

The initial recoveries by the mCP method from the freshwater suspensions were higher than those with the control spread plates, both aerobically and anaerobically. However, after 2 h of storage, the results were reversed; in fact, recoveries were not obtained by either method with the aerobically assayed dilutions. Again, no cells were recovered by either method after 24 h at the examined dilutions. These data suggest that: (i) even under the best of assay conditions, most of the *C. perfringens* vegetative cells are not recoverable by 24 h in either seawater or fresh water; (ii) the survival of vegetative cells is poorer in fresh water than in seawater; (iii) the combination of mCP medium and filtration reduces the recovery of "stored" but not "fresh" vegetative cells; (iv) this reduction is more pronounced when the cells are stored in fresh water; and (v) aerobic assay reduces the recovery of vegetative cells, especially those stored in fresh water for periods as short as 2 h. Furthermore,

it would appear that the aerobic assay does compound the inability of mCP to recover stored vegetative cells of this strain of *C. perfringens*.

In the second series of experiments, raw sewage was diluted, filtered, and incubated as described above, with one exception. For obvious reasons, the control assay in the anaerobic tent was conducted by using the mCP procedure. This was a compromise in that all the parameters were not controlled. The results of these experiments are shown in Table 4. It can be seen that the initial recoveries for both the anaerobic and aerobic conditions were approximately the same for both suspensions. The anaerobic recovery from the seawater suspension held for 24 h was approximately the same as the zero hour recovery, but the corresponding aerobic recovery was significantly lower. With the freshwater suspension, the recoveries after 24 h were approximately equal under both conditions; however, they were also about 23% lower than the respective recoveries at zero hour. The most likely explanation for these results is that (i) 20 to 30% of the *C. perfringens* population in the sewage sample were vegetative cells, (ii) because of factors in both sewage and seawater, they survive for periods up to 24 h in a sewage-sea-

TABLE 4. Comparative recoveries of *C. perfringens* from sewage suspensions under anaerobic and aerobic conditions

Suspension	Condition	No. of <i>C. perfringens</i> /unit vol after: ^a	
		0 h	24 h
Seawater	Anaerobic ^b	91	93
	Aerobic ^c	97	69
Fresh water	Anaerobic	105	78
	Aerobic	95	76

^a Values indicate average recovery from duplicate mCP filters.

^b Assay performed inside anaerobic tent.

^c Assay performed outside anaerobic tent.

TABLE 3. Comparative recoveries of *C. perfringens* vegetative cells (C-1861) from fresh- and seawater suspensions under anaerobic and aerobic conditions

Suspension	Condition	Recovery of vegetative cells per 100 ml by the following methods after: ^a					
		0 h		2 h		24 h	
		Control	mCP	Control	mCP	Control	mCP
Seawater	Anaerobic ^b	1.4×10^4	1.4×10^4	1.5×10^4	2.6×10^3	$<5.0 \times 10^2$	$<5.0 \times 10^0$
	Aerobic ^c	1.4×10^4	1.3×10^3	1.5×10^4	1.2×10^3	NT ^d	$<5.0 \times 10^0$
Fresh water	Anaerobic	1.8×10^4	3.9×10^4	6.5×10^3	5.0×10^1	$<5.0 \times 10^2$	$<5.0 \times 10^0$
	Aerobic	1.1×10^4	1.9×10^4	$<5.0 \times 10^2$	$<5.0 \times 10^0$	NT	$<5.0 \times 10^0$

^a Values indicate average recovery from duplicate control (TYI agar) spread plates and mCP membranes.

^b Assay performed inside anaerobic tent.

^c Assay performed outside anaerobic tent.

^d NT, Not tested.

water suspension, and (iii) even after storage in these suspensions for 24 h, they can be recovered by the mCP procedure, provided the assay is conducted under anaerobic conditions. However, when the sewage is diluted in freshwater and the suspensions are stored for 24 h, the vegetative cells therein cannot be recovered by the mCP method aerobically or anaerobically. In fact, they may not be recoverable at all.

The precision of the mCP method was determined from the D^2 values for assay variability calculated from the following equation as given by Eisenhart and Wilson (8): $D^2 = [n\sum Xi^2 - (\sum Xi)^2]/\sum Xi$, where $\sum Xi$ is the summation of the plate counts X_1, X_2, \dots, X_n , and n (the number of replicate plates per sample) was 5. The D^2 values calculated from the examination of 21 water samples from various locations are shown in Fig. 3 together with the expected D^2 control limits for $P = 0.005, 0.025,$ and 0.5 . The distribution of the D^2 values indicates that the counts obtained from the mCP method are randomly distributed and suggests that there is not a significant effect of location on plate-to-plate variability.

The counting range was determined as follows. Increasing quantities of the sample were assayed in quintuplicate. The mean *C. perfringens* density for each sample volume was determined. The expected values were obtained by

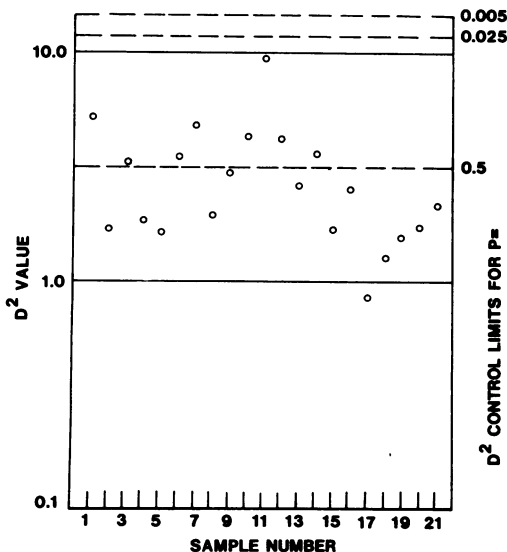


FIG. 3. Distribution of D^2 values. Fresh- and salt-water samples from various locations in New England were assayed in quintuplicate by the mCP method. The D^2 values were calculated as described in the text.

extrapolating from the mean count nearest 30 colonies per filter by using the following formula: $C_e = C_{30} V_e/V_{30}$, where C_e is the expected count for a given sample volume (V_e) examined, and C_{30} and V_{30} are the count nearest 30 colonies per filter and its corresponding volume. The observed and corresponding expected values for heated (60°C for 15 min) and unheated samples are plotted against each other in Fig. 4. As can be seen in this figure, the upper limit to the counting range for an unheated water sample appears to be 85 to 90 *C. perfringens* colonies per filter, whereas that for a heated sample is unaffected as high as 120 colonies per filter.

A further analysis of these same data allowed the counting range of the mCP method for both heated and unheated water samples to be evaluated as a function of its precision. Figure 5, which shows the precision of the method, indicates that the variability with both heated and unheated samples closely approximates that which is expected from random sampling error alone.

C. perfringens recoveries by mCP were compared to those by the BPT method, using heated and unheated samples from various locations in Rhode Island and Connecticut. The recommended heating regimen for each method was used, that is, 60°C for 15 min with mCP and 80°C for 5 min with BPT method. The density of *C. perfringens* for a given sample was calculated from the mean colony count as obtained from duplicate or triplicate filters and tubes (Table 5). The recoveries by the mCP method were comparable to or exceeded those by the BPT method in every instance.

From the greater destruction of spores with the heat treatment suggested by Bonde, as opposed to that with the treatment suggested for mCP (Fig. 2), it was expected that the BPT/mCP recovery ratios for the heated samples would have been markedly less than those for unheated samples. Such was the case for only six samples (no. 3, 4, 5, 9, 11, and 12). The notable exception was sample 1, whose BPT/mCP ratio markedly increased after the heat treatments. The high proportion of vegetative cells (Table 5) in this raw-sewage sample explains this finding. One explanation for the absence of a decrease in the BPT/mCP ratio with the heated portions of the remaining five samples (no. 2, 6, 7, 8, and 10) is that the portion of the spore population which was destroyed by heating at 80°C for 5 min also could not be recovered by the BPT method (Table 5).

DISCUSSION

The accuracy of the mCP method was satis-

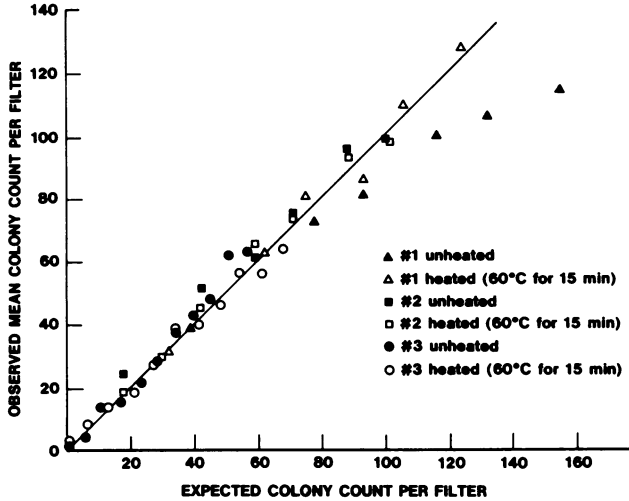


FIG. 4. Upper counting limit (maximum number of *C. perfringens* colonies per filter) for the mCP method, as determined from the loss, were assayed in quintuplicate. The observed values are the mean membrane filter counts; the expected values were derived as described in the text. Numbers 1 to 3 indicate samples collected from three different locations.

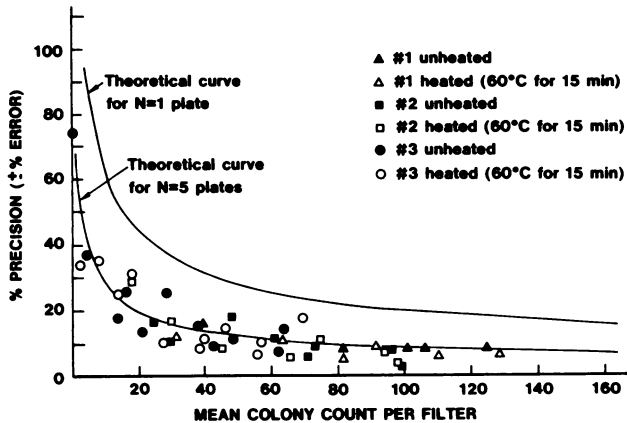


FIG. 5. Precision of the mCP method as a function of the counting range.

factory when evaluated against that particular performance criterion. However, there are a number of factors which limit the interpretation of data obtained. In the first place, the recoveries obtained were not absolute; rather, they were relative to those in a nutrient-rich control medium which contained no inhibitors. Natural samples would have provided more meaningful data; however, this would have presented the almost impossible logistical problem of picking and identifying colonies from the control medium. Natural samples were used in the comparison of recoveries by the mCP method with those by the BPT method, a part of the evaluation which will be discussed later.

Apparently, in mCP medium, germination of the spores occurs within a reasonable period of time, since the number of *C. perfringens* recovered from field samples did not increase with an increase in incubation time beyond 24 h. In addition, the requirement for heat activation of spores seems to have been obviated with the mCP method, since heating the samples at 60°C for periods of 5 to 30 min generally did not increase the number of *C. perfringens* recovered. In fact, more often than not, the number of *C. perfringens* decreased slightly after such treatments.

The specificity of the method was within the predetermined guidelines for the number of

TABLE 5. Comparative recoveries of *C. perfringens* from surface waters and sewage by the mCP and BPT methods

Sample no.	Sample location ^a	Type ^b	No. of <i>C. perfringens</i> recovered/100 ml ^c						Spores not recovered by BPT ^e	Heat-killed spores/
			No heat treatment			Heat treatment ^d				
			mCP	BPT	BPT/mCP	mCP	BPT	BPT/mCP		
1	Warwick STP, R.I.	RS	2.6 × 10 ⁵	6.3 × 10 ⁴	0.24	8.0 × 10 ⁴	6.1 × 10 ⁴	21	24	
2	Warwick STP, R.I.	PC	6.7 × 10 ⁴	2.2 × 10 ⁴	0.33	5.8 × 10 ⁴	1.9 × 10 ⁴	62	67	
3	Willimantic STP, Conn.	PC	3.6 × 10 ⁴	7.0 × 10 ²	0.02	3.1 × 10 ⁴	1.0 × 10 ²	98	98	
4	State Pier, R.I.	SW	6.9 × 10 ²	7.4 × 10 ²	1.07	5.9 × 10 ²	4.6 × 10 ²	—	22	
5	Squamum Woods, R.I.	SW	5.1 × 10 ²	3.4 × 10 ²	0.67	4.1 × 10 ²	7.4 × 10 ¹	17	82	
6	State Pier, R.I.	SW	7.3 × 10 ²	4.0 × 10 ²	0.55	6.2 × 10 ²	3.3 × 10 ²	35	47	
7	Fields Point, R.I.	SW	4.6 × 10 ³	1.8 × 10 ³	0.39	3.0 × 10 ³	1.1 × 10 ³	40	63	
8	Conimicut Point, R.I.	SW	8.0 × 10 ²	5.4 × 10 ¹	0.68	8.0 × 10 ¹	6.0 × 10 ¹	33	25	
9	Pawtuxet River, R.I.	SW	1.2 × 10 ³	6.2 × 10 ²	0.52	1.3 × 10 ³	4.5 × 10 ²	52	64	
10	Municipal Wharf, R.I.	SW	5.4 × 10 ³	2.3 × 10 ³	0.43	5.1 × 10 ³	2.5 × 10 ³	55	51	
11	R.I. Yacht Club	SW	5.7 × 10 ²	4.9 × 10 ²	0.86	6.3 × 10 ²	3.4 × 10 ²	21	46	
12	Willimantic, Conn.	FW	4.9 × 10 ²	3.4 × 10 ¹	0.07	4.9 × 10 ²	6.0 × 10 ⁰	31	99	
Avg										
						0.48			0.43	

^a STP, Sewage treatment plant.^b RS, Raw sewage; PC, prechlorination sewage; SW, seawater; FW, fresh water.^c Determined from triplicate filters (mCP) and tubes (BPT).^d Values indicate percent vegetative cells (mCP unheated - mCP heated/mCP unheated).^e Values indicate percentage of spores not recovered by BPT method (mCP heated - BPT heated/mCP heated).^f Values indicate percent killed by heating at 80°C for 5 min (mCP heated - BPT heated/mCP heated).^g Heat treatment for mCP was 60°C for 15 min, and that for BPT was 80°C for 5 min.

false-positive and false-negative colonies once IBDG was incorporated into the medium, as mentioned earlier. Colonial morphology was also important in the identification of *C. perfringens*. When sucrose fermentation is combined with acid phosphatase activity, the inability to ferment cellobiose, and growth at 45°C, *C. perfringens* can be separated from all but one other species of *Clostridium*, *C. pasteurianum* (4, 13, 24). However, *C. pasteurianum* apparently does not represent a problem with the mCP method, since this organism has not been identified as one producing a false-positive reaction. Although this particular organism has been found in soil (4), it is either present in waters in numbers so low as to be undetectable or it is unable to grow on mCP agar because of the inhibitors present. In spite of the acceptable specificity of the mCP method, verification of typical *C. perfringens* colonies is necessary when an operator is becoming accustomed to the procedure. In the absence of verification, the estimates of *C. perfringens* by the mCP method should be regarded as presumptive.

The distribution of the D^2 values indicates that the counts obtained by the mCP method are randomly distributed and that the sample location has little effect on the plate-to-plate variability.

The upper counting limit can be defined as the greatest number of colonies from a specified volume that can be counted on a single membrane filter without compromising the reliability of the method. The narrower counting range for unheated samples may have resulted from antibiotic produced by background colonies, inhibitors present in seawater, or competition for available nutrients. Whatever the explanation, heating the samples at 60°C for 15 min enhances the selectivity of the mCP procedure, as seen from the increase in the upper counting limit from 85 to more than 120 *C. perfringens* colonies. Over the counting range examined, plate-to-plate variability approximated that expected from random sampling and did not affect the upper counting limit. However, the combination of an upper counting limit of 85 colonies per filter and the especially poor precision when less than 20 colonies per filter are counted has methodological implications. Either half-log dilutions or triplicate assays are needed to provide reasonably reliable estimates.

The heating of samples to destroy vegetative cells is useful as a means of enhancing the selectivity of the mCP method and essential when *C. perfringens* spores are used as a conservative tracer. However, the temperature-time regimens used vary from author to author and include

temperatures between 70 and 85°C for periods from 5 to 30 min. The data obtained in the present study would suggest that these regimens, including that used by Bonde (80°C for 5 min), are too rigorous, since a large portion of the spore population is destroyed at times. In fact, even heating at 60°C for 15 min is a compromise between the required reduction in the number of vegetative cells on one hand and the unwanted destruction of heat-sensitive spores on the other. A better solution to this problem may be provided by an alternative treatment such as the use of ethanol (14).

Generally, the recoveries of *C. perfringens* by the mCP method were comparable to or exceeded those obtained by the BPT method. The density estimates obtained by either method were not confirmed as *C. perfringens*, and should be regarded as presumptive. However, if the average numbers of presumptive *C. perfringens* were adjusted by the respective verification frequencies for each method, the BPT/mCP recovery ratios would be even lower, since the confirmation frequency for mCP is 93% and that for the BPT method is about 79% (3). Nevertheless, we generally recovered twice as many *C. perfringens* with the mCP method as with the BPT method.

Recovery of *C. perfringens* vegetative cells by the mCP method is influenced by a number of factors, the most important of which is residence time and temperature within the environment. Another strong influence on recovery is the type of water in which the cells are present, since it was found in this study that survival of *C. perfringens* vegetative cells was poorer in fresh water than in seawater. Thus, to determine the numbers of *C. perfringens* vegetative cells by the mCP method, anaerobic assays are required and, even though, there may be a one- to two-log reduction in the recovery of "aged" cells attributable to the combination of the medium and membrane filtration. It would seem that *C. perfringens* at least survives better in seawater than in fresh water.

Heat-sensitive *C. perfringens* spores were detected less often than anticipated, particularly in samples from upper Narragansett Bay, R.I., an area impacted with a number of pollution sources. One could speculate that these spores are so heat sensitive that they are not only destroyed by the Bonde temperature-time regimen, but also by that recommended for the mCP method. If such is the case, then alternative methods (e.g., ethanol treatment) (14) should be applied when the numbers of vegetative cells are to be determined.

The availability of a relatively facile method

which enumerates *C. perfringens* specifically should further a reexamination of the value of this fecal indicator system.

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