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An American National Standard

Standard Test Method for Detection and Enumeration of Clostridium perfringens from Water and Extracted Sediments by Membrane Filtration (MF)[1]

This standard is issued under the fixed designation D 5916; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method can enumerate *Clostridium perfringens* spores and vegetative cells from marine water, sediment, wastewater, ambient water, and drinking water. Since *C. perfringens* spores are present in large numbers in human and animal wastes and are resistant to wastewater treatment practices, extremes in temperature, and environmental stress, they are an indicator of present fecal contamination as well as a conservative tracer of past fecal contamination. It is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

1.2 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water[2]
D 1193 Specification for Reagent Water[2]
D 1888 Methods of Test for Particulate and Dissolved Matter in Water[3]
D 2777 Practice for Determination of Precision and Bias of Applicable Methods of Committee D19 on Water[2]
D 3370 Practices for Sampling Water from Closed Conduits[2]
D 3863 Test Method for Retention Characteristics of 0.4 to 0.45-μm Membrane Filters Used in Routine Filtration Procedures for the Evaluation of Microbiological Water Quality[4]
D 3870 Practice for Establishing Performance Characteristics for Colony Counting Method in Bacteriology[4]
D 5465 Practice for Determining Microbial Counts from Waters Analyzed by Plating Methods[4]
E 200 Practice for Preparation, Standardization, and Storage of Standard and Reagent Solutions for Chemical Analysis[5]

3. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminology D 1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *Clostridium perfringens—in this test method, C. perfringens* is defined as an obligate anaerobic gram-positive, spore forming, nonmotile bacillus, 0.9–1.3 by 3.0–9.0 μm in size that ferments sucrose, ferments lactose with stormy gas production, does not ferment cellobiose, and produces acid phosphatase. *Clostridium perfringens* also produces toxins which cause gas gangrene and gastroenteritis.

3.2.2 *spores—C. perfringens* produces single oval subterminal spores less than 1.0 μm in diameter during adverse conditions. Sporulation can also occur in the intestinal tract. The endospore that develops is a highly refractile body formed within the cell. Spores are notably resistant to heat, drying, and chemical disinfectants which would kill the vegetative forms of *C. perfringens*. This resistance to unfavorable conditions preserves the organisms for long periods of time.

4. Summary of Test Method

4.1 Appropriate volumes of water are passed through membrane filters (MF) that retain the bacteria present in the sample. The MFs are placed on mCP agar modified by Armon and Payment (**1**) [6] from the medium of Bisson and Cabelli (**2**) and are incubated anaerobically at 44.5°C for 24 h. The yellow, straw-colored *C. perfringens* colonies which turn dark pink to magenta on exposure to ammonium hydroxide are counted and reported *C. perfringens* colony forming units (CFU) per 100 mL. Because of the selectivity of the mCP medium, presumptive counts are normally reported for routine monitoring purposes. If verification is desired, colonies are confirmed by

[1] This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.
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[2] *Annual Book of ASTM Standards*, Vol 11.01.
[3] Discontinued; see *1989 Annual Book of ASTM Standards*, Vol 11.01.
[4] *Annual Book of ASTM Standards*, Vol 11.02.
[5] *Annual Book of ASTM Standards*, Vol 15.05.
[6] The boldface numbers in parentheses refer to the list of references at the end of this test method.

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anaerobic growth in thioglycollate, a positive gram stain reaction and stormy fermentation of iron milk, and mCP counts adjusted based on the percent confirmation.

4.2 For sediment analyses, 1 to 10 g of wet sediment is weighed, water added, and mixed by vortex and sonication. After settling, the water layer is analyzed as described in 4.1.

4.3 Verification of counts is not required. However, if verification is desired, colonies can be confirmed by anaerobic growth in thioglycollate, a positive gram stain reaction and stormy fermentation of iron milk. The mCP counts may be adjusted based on the percent confirmation.

5. Significance and Use

5.1 *Clostridium perfringens* is a strict obligate anaerobe that is found in fecal material. Under moderately adverse conditions these organisms produce endospores that can withstand extreme environmental conditions and are conservative tracers of past and present pollution in fresh and marine waters and sediments.

6. Interferences

6.1 Waters containing sediment, large quantities of colloidal or suspended materials such as iron, manganese, alum floc, or algae can clog the filter pores and prevent filtrations or cause the development of spreading bacterial colonies which may mask target colonies and prevent accurate counting.

6.2 When bacterial densities are high, a smaller sample volume or sample dilution can be filtered to minimize the interference of turbidity or high background (nontarget) bacterial densities. Replicates of smaller sample volumes or dilutions of sample may be filtered and the results combined. However, the membrane filter techniques may not be applicable to highly turbid waters with low *Clostridium* densities.

6.3 Toxic materials such as metals, phenols, acids, caustics, chloramines, and other disinfection by-products may also adversely affect recovery of *Clostridium* vegetative cells on the MF. The most probable number (MPN) method should be considered as an alternative procedure for these samples **(3)**. Use with discretion.

7. Apparatus

7.1 *Microscope*, stereoscopic, wide-field type, with magnification of 10 to 15×.

7.2 *Microscope Lamp*, that produces diffuse light from a cool white fluorescent or tungsten lamp adjusted to give maximum visibility.

7.3 *Counting Device*, hand tally or electronic.

7.4 *Pipet Container*, stainless steel or aluminum, for sterilization and storage of glass pipets.

7.5 *Pipets*, sterile T.D. bacteriological or Mohr, glass or plastic, of appropriate volume.

7.6 *Graduated Cylinders*, 100 to 1000 mL, covered with aluminum foil or kraft paper and sterilized.

7.7 *Membrane Filtration Units* (filter base and funnel), glass, plastic, or stainless steel, wrapped with aluminum foil or kraft paper and sterilized.

7.8 *Ultraviolet Unit*, for disinfecting the filter funnel between filtrations (optional).

7.9 *Line Vacuum*, electric vacuum pump or aspirator as a vacuum source. In an emergency or in the field, a hand pump or a syringe can be used if equipped with a check valve to prevent the return flow of air.

7.10 *Flask, Vacuum*, usually 1 L, with appropriate tubing. Filter manifolds to hold a number of filter bases are optional.

7.11 *Flask, Safety Trap*, placed between the filter flask and the vacuum source.

7.12 *Forceps*, straight or curved, with smooth tips to permit handling of filters without damage.

7.13 *Thermometers*, 0 to 50 ± 0.2°C and 0 to 100 ± 0.5°C, which have been checked against a National Institute of Standards and Technology (NIST) certified thermometer or against one traceable to NIST.

7.14 *Petri Dishes*, sterile, plastic or glass, 50 by 9 mm with tight-fitting lids, or 60 by 15 mm with loose-fitting lids.

7.15 *Bottles*, milk dilution, borosilicate glass or plastic, screw-cap with neoprene liners, marked at 99 mL for 1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10 dilutions.

7.16 *Test Tubes*, 150 by 20 mm, borosilicate glass or disposable plastic.

7.17 *Caps*, aluminum or autoclavable plastic, for 150 by 20 mm test tubes.

7.18 *Centrifuge Tubes*, 50 mL, polycarbonate.

7.19 *Inoculation Loops*, (3 mm diameter) and needles, nichrome or platinum wire, 26 B & S gage, in suitable holders. Disposable applicator sticks or plastic loops are acceptable alternatives to inoculation loops.

7.20 *Waterbaths*, that maintain 46 to 48°C for tempering agar and 60 ± 0.5°C for heat shock.

7.21 *Sonication Unit*, to break up soil and sediment particles prior to analyses. [7]

7.22 *Anaerobic System* (anaerobic jar, reaction chamber, hydrogen/carbon dioxide disposable generator and anaerobic indicator), or any other system capable of producing the appropriate anaerobic conditions to support the growth of the organisms. [8]

7.23 *Incubator*, with temperature capability of 44.5 ± 0.2°C, large enough to hold the anaerobic system.

7.24 *Filter Paper*, circular, 11 cm, Whatman 40 or 110, or equivalent, for separation of mCP agar during anaerobic incubation.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where

[7] Bronson Sonifier, 500 W, or Tekmar Sonic Disrupter, 500 W with 3 mm tip set at 18 W, or equivalent.

[8] BBL 60460 or BBL 60466 GasPak Anaerobic System with BBL 70308 Disposable Hydrogen and Carbon Dioxide Generator Envelopes, BBL Microbiological Systems, Cockeysville, MD 21030, or equivalent.

such specifications are available.[9] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. Use microbiological grade agar in preparation of culture media. Whenever possible, use commercial culture media as a means of quality control.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type II of Specification D 1193.

8.3 *Ethanol 95 %, Pure*, for preparation of acetone alcohol and for flame-sterilization of forceps.

8.4 *Membrane Filters*, sterile, white, grid marked, 47 mm diameter, with 0.45 ± 0.02 µm pore size or other pore sizes for which the manufacturer provides data demonstrating equivalency.

8.5 *Buffered Dilution and Rinse Water*:

8.5.1 *Stock Phosphate Buffer Solution*—Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL of water. Adjust pH to 7.2 with 1 *N* NaOH and bring to 1000 mL with water. Dispense aseptically into screw-cap bottles and autoclave at 121°C for 15 min. Alternatively, sterilize by filtration through a 0.2 µm pore membrane filter and dispense aseptically in sterile screw-cap bottles. Store in refrigerator and handle aseptically. If cloudiness, a marked change in pH, or other evidence of contamination appears, discard the stock. Confirm that pH is 7.2 ± 0.5 before use.

8.5.2 *Magnesium Chloride Solution*—Dissolve 81.4 g of hexahydrate magnesium chloride ($MgCl_2 \cdot 6H_2O$) in 1000 mL of water. Mix well and sterilize by filtration or autoclave at 121°C for 15 min. Store in refrigerator and handle aseptically. If cloudiness or other evidence of contamination occurs, discard the stock solution.

8.5.3 *Phosphate Buffered Dilution Water*—Add 1.25 mL of stock phosphate buffer solution and 5 mL of magnesium chloride solution to 1000 mL of water in a volumetric flask and mix well. Dispense dilution water in amounts which will provide 99 ± 2 mL after sterilization in screw-cap dilution bottles, or in larger volume containers for use as rinse water if desired. Autoclave immediately at 121°C for 15 min. Autoclave larger volumes for longer periods as appropriate. Alternatively, sterilize by filtration through a 0.2 µm pore membrane filter and dispense aseptically into sterile screw-cap bottles.

8.6 *Ferric Chloride Solution*—Weigh out 4.5 g of $FeCl_3 \cdot 6H_2O$ and dissolve in 100 mL of water. Filter, sterilize, and store in refrigerator.

8.7 *Phenolphthalein Diphosphate Solution*—Weigh out 0.5 g of phenolphthalein diphosphate and dissolve in 100 mL of water. Filter, sterilize, and store in refrigerator.

8.8 *Indoxyl β-D Glucoside Solution*—Weigh out 0.06 g of indoxyl β-D glucoside and dissolve in 80 mL of water (0.075 % solution). Sonicator in 7.21 can be used to speed dissolution. Filter, sterilize, and use in 8.9.2.

8.9 *mCP Agar, (modified)* **(1)**.

8.9.1 *Composition/Litre*:

Tryptose	30.0 g
Yeast extract	20.0 g
Sucrose	5.0 g
L-cysteine	1.0 g
$MgSO_4 \cdot 7H_2O$	0.1 g
Bromcresol purple	0.04 g
Agar	15.0 g

8.9.2 *Preparation*—In a 1000 mL Erlenmeyer flask add medium ingredients from 8.9.1 to 900 mL water, stir, and heat to dissolve. Bring the pH to 7.6 with 1 *N* NaOH. Autoclave for 15 min at 121°C (15 lb pressure). Cool to 50°C. Add the following reagents aseptically and mix well:

D-cycloserine	0.4 g
Polymyxin B sulfate	0.025 g
4.5 % $FeCl_3 \cdot 6H_2O$ solution	2.0 mL
0.5 % Phenolphthalein diphosphate solution	20.0 mL
0.075 % Indoxyl-β-D-glucoside solution	80.0 mL

8.9.3 Dispense 4 to 4.5 mL into each 50 by 9 mm petri plate using a sterile Cornwall syringe or Brewer pipette and allow to harden. Store agar inverted in a sealed plastic bag in a refrigerator at 4°C for no more than one month. It is recommended that the agar plates be stored in an anaerobic chamber in the refrigerator if possible.

8.10 *Iron Milk Medium*.[10]

8.10.1 *Composition/Litre*:

Fresh whole milk	1 L
Ferrous sulfate·$7H_2O$	1 g

8.10.2 *Preparation*—Dissolve ferrous sulfate in 50 mL water. Add slowly to 1 L milk and mix with magnetic stirrer. Dispense 11 mL medium into culture tubes. Autoclave at 118°C for 12 min.

8.11 *Fluid Thioglycollate Medium*.[11]

8.11.1 *Composition/Litre*:

L-cysteine	0.5 g
Agar (granulated)	0.75 g
NaCl	2.5 g
Dextrose (anhydrous)	5.0 g
Yeast extract	5.0 g
Tryptone	15.0 g
Sodium thioglycollate	0.5 g
Resazurin	0.001 g

8.11.2 *Preparation*—Suspend 29.5 g of medium in 1 L of water. Mix thoroughly and heat to boil for 1 to 2 min or until solution is complete. Final pH is 7.1 ± 0.1. Dispense 15 mL portions into culture tubes. Cap and autoclave at 121°C for 15 min. Store tubes in the dark at room temperature. Do not refrigerate. If medium becomes oxidized (if more than 30 % of medium is pink), reheat once only in boiling water bath and cool before use.

8.12 *Gram Stain Reagents*:

[9] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

[10] *FDA Bacteriological Analytical Manual*, 7th Ed., AOAC International, Arlington, VA, 1992, pp. 476–477, iron milk medium (modified).

[11] *Fluid Thioglycollate Medium* (BBL 11259), Benton-Dickinson Microbiology Systems, Cockeysville, MD; (Difco 0432-02-6), Difco Laboratories, Detroit, MI, or equivalent.

8.12.1 Gram stain reagent kits are commercially available and are recommended.

8.12.2 *Ammonium Oxalate-Crystal Violet (Hucker's)*—Dissolve 2 g crystal violet (90 % dye content) in 20 mL 95 % ethyl alcohol. Dissolve 0.8 g $(NH_4)_2C_2O_4H_2O$ in 80 mL water; mix the two solutions and age for 24 h before use. Filter through paper into a staining bottle.

8.12.3 *Lugol's Solution, Gram's Modification*—Grind 1 g iodine crystals and 2 g KI in a mortar. Add water, a few millilitres at time, and grind thoroughly after each addition until solution is complete. Rinse solution into an amber glass bottle with the remaining water (using a total of 300 mL).

8.12.4 *Counterstain*—Dissolve 2.5 g safranin dye in 100 mL 95 % ethyl alcohol. Add 10 mL to 100 mL water.

8.12.5 *Acetone Alcohol*—Mix equal volumes of ethyl alcohol (95 %) with acetone.

9. Hazards

9.1 The analyst/technician shall know and observe the normal good laboratory practices and safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials and while operating sterilizers and other equipment and instrumentation.

9.2 Field and laboratory staff who collect and analyze environmental samples are under some risk of exposure to pathogenic microorganisms. Therefore, staff should always apply safety procedures used for pathogens to all samples. See CDC's *Biosafety in Microbiological and Biomedical Laboratories* (**4**).

9.3 Do not pipet by mouth.

10. Sampling

10.1 Collect water samples in sterile, nontoxic glass or plastic containers with leak-proof lids. Collect 10 to 20 g sediment samples and place in a sterile 4.5 oz plastic cup or other appropriate sterile container with leak-proof lid.

10.2 Use insulated containers to maintain water samples on ice or refrigerate at a temperature of 1 to 4°C during transit to the laboratory. Take care that sample container tops and closures are not submerged in water during transit or storage. Refrigerate samples upon arrival in the laboratory and analyze as soon as possible after collection.

10.3 Although *C. perfringens* vegetative cells are sensitive to aerobic conditions and are not expected to survive well in storage, *C. perfringens* spores can survive for extended periods at 1 to 4°C. However, if a correlation is planned with other indicator or pathogenic microorganisms, the holding time for *C. perfringens* should be limited to that of the other organisms.

10.4 Sampling procedures are described in Practices D 3370. Adherence to sampling procedures, preservation procedures, and holding time limits is critical to the production of valid data. Reject samples if appropriate sampling, preservation, and handling procedures have not been followed.

11. Quality Control

11.1 Check and record temperatures in incubators daily to ensure operation within stated limits.

11.2 Check thermometers and record the results at least annually against a National Institute of Standards and Technology (NIST) certified thermometer or one traceable to NIST. Record results. Check mercury columns for separation.

TABLE 1 Sample Volumes to Obtain Colony Count on Membrane Filters

Range of 20 to 80 Colonies	
Sample vol in mL	Added as:
0.05	5.0 mL of 10^{-2}
0.20	2.0 mL of 10^{-1}
0.80	8.0 mL of 10^{-1}
3.2	3.2 mL undiluted sample
15.0	15.0 mL undiluted sample
60.0	60.0 mL undiluted sample

11.3 As a quality control over anaerobic conditions, temperature, and media, spot test a separate mCP agar plate with a pure culture of *C. perfringens* and include in each test run. Examine for the appropriate response.

11.4 For general quality control recommendations, see" Quality Assurance for Microbiological Analyses of Water" in *ASTM Special Technical Testing Publication 867* (**5**).

12. Procedure for Water Samples

12.1 Prepare mCP Agar according to 8.9.

12.2 Select sample volumes based on previous knowledge to produce membrane filters with 20 to 80 *C. perfringens* colonies. Sample volumes and dilutions are selected to provide a dilution factor of 4 or 5 among them. An example of such factors is shown in Table 1.

12.2.1 The range of volumes and dilutions selected for filtration of completely unknown samples can be broader to provide a dilution factor of 10 or more. Prepare at least three sample increments. If confluent growth is encountered or the number of CFUs exceed 80, repeat analysis to obtain a countable range.

12.3 Mark petri dishes and laboratory data sheets with sample identities and volumes.

12.4 Grasp a sterile membrane filter by its edge using sterile forceps and place on the filter base, grid side up. Attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and the base.

12.5 Shake the sample bottle vigorously about 25 times and measure the desired volume of sample into the funnel with the vacuum off. To measure the sample accurately and obtain good distribution of colonies on the filter surface, use the following procedures:

12.5.1 *Sample Volumes of 20 mL or More*—Measure the sample in a sterile graduated cylinder and pour it into the funnel. Rinse the graduate twice with sterile dilution water and add the rinse water to the funnel.

12.5.2 *Sample Volumes of 10 to 20 mL*—Measure the sample with a sterile 10-mL or 25-mL pipet into the funnel.

12.5.3 *Sample Volumes of 1 to 10 mL*—Pour about 20 to 30 mL of sterile dilution water into the funnel without vacuum and add the sample to the sterile water using appropriate sterile pipet.

12.5.4 *Sample Volumes of Less Than 1.0 mL*—Prepare appropriate dilutions in sterile dilution water and proceed as applicable in step 12.5.3 above.

12.5.5 The time elapsing between preparation of sample dilutions and filtration should be minimal and never more than 30 min.

12.6 For greatest accuracy, it is desirable to filter the largest possible sample volume. However, if past analyses of specific samples have resulted in confluent growth or "too numerous to count" (TNTC) membranes from excessive turbidity, smaller volumes or greater dilutions should be used.

12.7 When analyzing a series of samples or dilutions, filter samples in order of increasing volumes of original sample.

12.8 After adding the sample to filter funnel, turn on vacuum and filter the sample. Rinse the sides of the funnel walls at least twice with 20 to 30 mL of sterile dilution water. Turn off vacuum and remove the funnel from the filter base.

12.9 Flame forceps, cool, and aseptically remove the MF from the filter base. Place the filter, grid side up, on the agar using a rolling motion to prevent air bubbles. Reseat the filter if bubbles occur.

12.10 Remove the lids from the mCP Agar and place each lid under the corresponding plate bottom for identification. Invert and stack the open plates in layers in the anaerobic chamber, separating each layer with sterile filter paper. Incubate the anaerobic chamber at 44.5°C for 24 h. Anaerobic conditions are maintained through the use of a commercial anaerobic system. If visible condensation does not occur within 60 min after the BBL GasPak is activated, terminate the reaction by opening the jar and removing the GasPak. Inspect the chamber seal for alignment and lubricant. Insert a new GasPak and seal the chamber. The disposable anaerobic indicator (moistened flat fiber wick impregnated with 0.35 % methylene blue solution) is white to pale blue upon opening foil envelope. It turns blue upon exposure to air. Under anaerobic conditions the methylene blue indicator becomes decolorized within 2 to 4 h and turns white. It should remain white through the incubation period.

12.11 After 24 h, remove one agar plate at a time from the chamber and reclose the chamber. Examine the plate for yellow, straw-colored colonies. If yellow colonies are present, invert and expose the open plate for 10 to 30 s to the fumes of an open container of concentrated (29.2 %) ammonium hydroxide. Avoid inhalation of fumes.

12.12 If *C. perfringens* is present, the phosphate in the phenolphthalein diphosphate is cleaved from the substrate by acid phosphatase and typical colonies of *C. perfringens* turn a dark pink or magenta after exposure to fumes of ammonium hydroxide. Count pink or magenta colonies as presumptive *C. perfringens*.

12.13 Repeat steps 12.11 and 12.12 for other cultures.

13. Procedure for Sediment Samples (Sediment Extraction)

13.1 Determine the dry weight of the sediment according to Test Method D 1888, Test Method A, Total Matter.

13.2 Prepare mCP agar according to 8.9.

13.3 Weigh out 0.1 to 10.0 g of the original wet sediment into a sterile 50 mL centrifuge tube. Add 10 mL of sterile water and vortex 10 to 15 s on high speed.

13.4 Sonicate with sonicator set at 5 (output control), 50 % duty cycle for intermittent operation (pulse), time and hold; count 20 pulses (bursts).

13.5 Add 25 mL of sterile reagent water, and vortex 10 to 15 s and let mixture settle 10 min or more.

13.6 Follow the procedural steps in 12.2-12.13.

13.7 Adjust the *C. perfringens* CFU count based on the results in 13.1 and report the CFU count per gram of sediment, dry weight.

14. Procedure for Enumeration of Spores Only

14.1 To obtain a count of *C. perfringens* spores only, hold water or water extracted from sediment samples in 60°C waterbath for 15 min to kill all vegetative cells. The analyst is reminded that heat can destroy heat-sensitive spores and reduce the spore count.

14.1.1 Equilibrate a waterbath at 60°C.

14.1.2 Determine the time necessary to bring a blank sample to 60°C.

14.1.3 Immerse sample containers in waterbath for the time necessary to warm sample to 60°C plus 15 min. Do not allow the container cap or opening to become contaminated by waterbath water.

14.1.4 To reduce the total time needed to heat-shock in 14.1.3, heat the samples in the smallest practical volumes in thin-walled glass containers.

14.1.5 Proceed with the analyses in Section 12 or 13.

15. Confirmation Tests

15.1 Pick at least 10 typical isolated *C. perfringens* colonies from the mCP plate and transfer each into a separate thioglycollate tube. If less than 10 colonies are present, pick all that are typical. Incubate at 35°C for 24 h. Examine by gram stain and for purity. *Clostridium perfringens* are shortgram-positive bacilli. Retain tubes for further testing.

15.2 Inoculate tubes of iron milk media with 1 mL from each of the ten fluid thioglycollate tubes and incubate in a 44.5°C waterbath for 16 to 18 h. Examine periodically for stormy fermentation with rapid coagulation and fractured rising curd.

15.3 Those colonies which are gram-positive, nonmotile, and produce stormy fermentation of milk in these confirmatory tests are considered confirmed *C. perfringens*.

16. Calculations and Reporting Results

16.1 Pink or magenta colonies counted on mCP medium are adjusted to a count per 100 mL and reported as presumptive *C. perfringens* colony forming units (CFU) per 100 mL.

16.2 If confirmation tests are performed, original counts on mCP agar are adjusted based on the percent of colonies picked and confirmed. Report as confirmed *C. perfringens* CFU per 100 mL of water sample or CFU per gram sediment sample, dry weight.

TABLE 2 Results of *Clostridium perfringens* Research Report Study, in CFU/g Dry Wt for Sample 1; CFU/100 mL for Samples 2–5

Analyst	Sample: 1			2			3			4			5		
	REP			REP			REP			REP			REP		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	3333	2820	3487	124	116	152	108	72	104	7000	7000	12000 [A]	96	72	88
2	4102	3179	3589	95	115	170	80	90	50	5500	7500	7600	65	90	65
3	3025	3128	3948	133	120	133	60	87	100	7250	6500	6750	66	60	107
4	2359	2871	2307	100	115	115	35	65	50	6800	5900	5500	60	110	75
5	3692	3179	3179	90	85	90	...	...	...	6000	6800	5000	85	85	65
6	2974	2666	2564	107	93	120	83	70	57	7900	8100	5800	27	27	47
7	2359	3692	2974	77	110	133	47	47	37	4800	5800	6500	30	20	40
8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
9	...	...	...	100	120	220 [A]	...	...	...	2000	9500	6400	...	...	...
10	1564	1615	1846	110	110	120	55	105	90	2800	3200	4100	110	60	110
11	...	...	...	60	65	100	95	85	65	3600	4100	5900	...	...	...
12	2769	3743	3640	145	140	100	85	60	50	5600	7400	5900	40	45	70
13	2051	2000	2154	80	47	93	...	...	...	5700	6400	6900	...	...	...
14	...	...	...	...	...	...	120	50	90	...	...	...	...	...	...

[A] Outlier.

17. Precision and Bias [12]

17.1 The minimum detection limit is one CFU per volume of sample or sample dilution tested.

17.2 The confirmation rate of this test method is reported to be 93 % (**2**).

17.3 *Round Robin Study*:

17.3.1 Sixteen analysts from nine laboratories analyzed a sediment, an unchlorinated wastewater, and three spiked waters (marine water, lake water, and a finished drinking water) as unknowns. Analysts were provided range values to reduce the number of dilutions necessary for the analyses.

17.3.2 Of the 16 data sets, three sets were rejected out of hand because the labs either did not follow method instructions for the study or did not correctly report results. Other data sets for some samples were rejected because the laboratories used dilutions which produced results falling outside the desired counting range of approximately 15 to 80 CFU/agar plate. The triplicate plate counts were averaged and the average counts used to calculate the reported colony forming units (CFU) are shown in Table 2. The blanks in Table 2 represent the unusable data. The remaining data were judged acceptable for evaluation and are tabulated as shown.

17.3.3 In Table 3, the single-operator precision (as % RSD) ranged from 14 to 28 % while the overall precision (as % RSD) ranged from 24 to 41 %, for S_t/S_o ratios of 1.13 to 1.80. The larger RSD values were not generated with the more difficult sample matrices of sediment and wastewater. Rather, they occurred with the seeded finished drinking water sample and are believed to have been caused by overestimates of the concentration of *C. perfringens*, which resulted in marginally low plate counts with inherently greater deviations. Overall, the S_o and S_t values were quite similar across sample types and concentration levels of *C. perfringens*. A comparison of S_o and S_t values across all sample types is shown in Fig. 1.

17.3.4 Although there were no “standards” available for this research report study, Sample 5, a seeded drinking water, had a reference count of 78 *C. perfringens* CFU/100 mL. The laboratories in this study achieved a mean recovery of 67 CFU from Sample 5 for a percent recovery of 86 %.

18. Keywords

18.1 anaerobic bacteria; *Clostridium*; *Clostridium perfringens*; indicator organisms; pollution; spore-forming bacteria; water quality

[12] Supporting data are available from ASTM Headquarters. Request research report RR:D19-1155.

TABLE 3 Statistical Evaluation of Results, in CFU/g Dry Wt or CFU/100 mL (After Rejection of Outliers)

Sample	Initial, *n*	Final, *n*	$\bar{X}$	S_o	S_t	%RSD (S_o)	%RSD (S_t)
1	30	30	2893.63	397.78	715.45	13.75	24.73
2	36	35	108.09	20.34	26.18	18.82	24.22
3	30	30	73.07	20.29	23.23	27.77	31.79
4	36	35	5985.71	1400.70	1585.80	23.40	26.49
5	27	27	67.22	18.64	27.60	27.73	41.06

Percent Recovery on Sample #5, Seeded Finished Water Sample

Reference Value = 78 CFU/100 mL
Mean Recovery Value = 67 CFU
Percent Recovery = 86 %

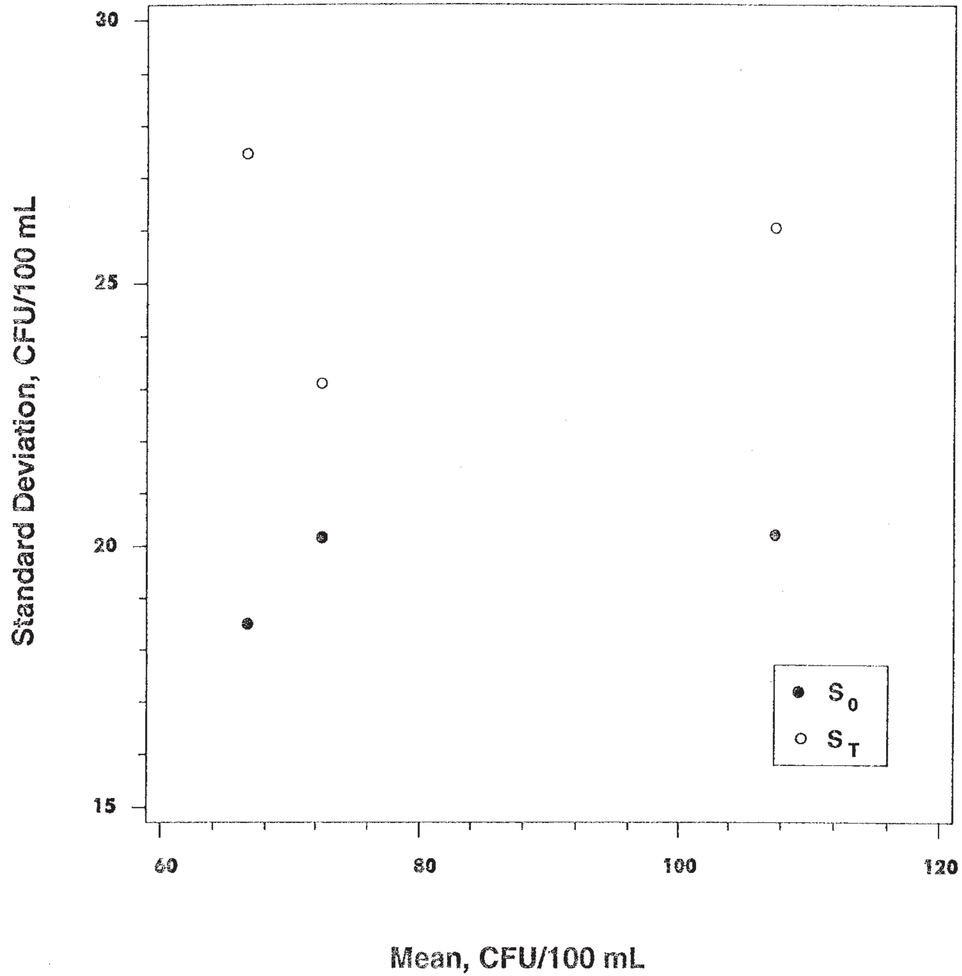

FIG. 1 Overall and Single-Operator Standard Deviations Against Mean

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