Section E

MICROBIOLOGICAL EXAMINATION

TABLE OF CONTENTS

SECTION E Microbiological Examination

Coliform Presence-Absence (P-A) Test in Drinking Water, Fresh Water, and Finished Water

Parameter Coliform, Presence-Absence

Analytical Method and EMS Codes **COLI X386**

Scope This method describes the enrichment culture of large volumes of water expected to be devoid of coliforms in fresh water, or other finished water systems. This method is not influenced by turbidity. In the event of a positive P-A test, subsequent samples must be analyzed by the membrane filter or MPN technique until two consecutive samples yield negative tests.

Principle The presence-absence test is a modification of existing procedures which can be used to monitor water systems that are normally expected to be free of coliforms, such as drinking water or other finished water systems. This test is not quantitative, and positive tests must be followed by subsequent samples analyzed by either membrane filtration or MPN until the bacterial counts fall below the detectable level. The advantages of the presence-absence test are cost-efficiency of testing normally coliform-free sites, and the capability of differentiating total coliforms, fecal coliforms, and fecal streptococci by subsequent culture on differential media. The P-A test may also maximize recovery of stressed organisms which may be missed in routine coliform testing. Regulations which stipulate an absence of coliforms in a 100mL sample can be addressed by P-A since the absolute number of organisms greater than unity is unimportant.

Sample Handling The sample is collected in the field and submitted unfiltered and unpreserved in a sterilized water bacteriology bottle. Chlorinated water samples should be treated with sodium thiosulfate (final concentration 0.01% w/v) to neutralize the bactericidal effect of chlorine. The sample should be kept at 4°C until analysis. Variations in temperature are to be avoided. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum volume required for analysis is 100mL.

Range Positive/Negative

Detection Limit Negative/100mL.

Interferences Residual chlorine in chlorinated water must be neutralized with sodium thiosulfate at time of sampling.

Precision There are no standard reference materials for the P-A test. American Type Culture Collection (ATCC®) bacterial cultures can be used to test recovery and performance of media. Recommended cultures are: ATCC 23355 Enterobacter aerogenes, ATCC 25922 Escherichia coli, ATCC 29212 Streptococcus fecalis.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 35± 0.5°C temperature.
b) Lauryl tryptose broth or lauryl sulfate broth.
c) Lactose broth.
d) Lauryl tryptose broth with MUG or lauryl sulfate broth with MUG.
e) Azide dextrose broth.
f) Bile esculin agar.
g) 18mm test tubes with inverted fermentation vials or Durham tubes and stainless steel closures.
h) Autoclavable media bottles, 250mL.
i) Autoclave for steam sterilization of glassware and media.
j) Cylinders, graduated, glass, 100mL covered with kraft paper or aluminum foil and sterilized.
k) Bunsen burner.
l) Platinum inoculation loops, 3mm diameter.
m) Microscope slides and microscope with oil immersion lens.
n) Sterile disposable petri plates, 100 x 15mm.
o) Deionized or distilled water meeting the criteria of reagent grade water as specified in Section 9020:I Standard Methods for the Examination of Water and Wastewater [a].

Reagents

a) P-A COLIFORM BROTH.

Lactose broth	39.000 g
Lauryl tryptose broth	52.500 g
Bromcresol purple (CAS 115-40-2)	0.0255 g
Deionized or distilled water (DI)	1.000 L

Add the lactose broth and lauryl tryptose broth sequentially to the water, stirring to dissolve. Dissolve the bromcresol purple in 10mL 0.1N NaOH and add to the broth solution. Dispense 50mL aliquots into 250mL media bottles. Autoclave for 12min at 121°C. Do not overheat or prolong cycle. Finished medium pH should be 6.8 ± 0.2.

b) LAURYL TRYPTOSE BROTH WITH MUG (DIFCO)

Formula (grams per litre):

Bacto tryptose	20.00 g
Bacto lactose	5.00 g
Potassium phosphate dibasic	2.75 g
Potassium phosphate monobasic	2.75 g
Sodium chloride	5.00 g
Sodium lauryl sulfate	0.10 g
MUG (4-methylumbelliferyl -B-D-glucuronide)	0.05 g

Suspend all ingredients in 1L DI and warm slightly to dissolve completely. Dispense 10mL aliquots into 18mm test tubes with inverted fermentation vial (Durham tube) in each tube. Place 18mm stainless steel closures on tubes and sterilize in autoclave for 15 minutes at 121°C using the liquid cycle. Do not fully open autoclave door until chamber temperature has dropped below 75°C to avoid trapping air bubbles in the inverted vials. Final pH of the medium is 6.8 at 25°C.

c) AZIDE DEXTROSE BROTH (DIFCO)

Formula (grams per litre):

Bacto beef extract	4.5 g
Bacto tryptose	15.0 g
Bacto dextrose	7.5 g
Sodium chloride	7.5 g
Sodium azide*	0.2 g

Suspend all ingredients in 1L DI and warm slightly to dissolve completely. Dispense 10mL aliquots into 18mm test tubes. Place 18mm stainless steel closures on tubes and sterilize in autoclave for 15 minutes at 121°C. Final pH of the medium is 7.2 at 25°C.

***Note**: Sodium azide is a potent neurotoxin. Avoid breathing dust. Avoid contact with eyes, skin, and clothing. Do not allow azide to come in contact with metal drain pipes. Flush with copious amounts of water when discarding down drains.*

d) BILE ESCULIN AZIDE AGAR, dehydrated (DIFCO)

Formula (grams per litre):

Bacto beef extract	5.00 g
Proteose peptone no. 3	3.00 g
Bacto tryptone	17.00 g
Bacto oxgall	10.00 g
Bacto esculin	1.00 g
Ferric ammonium citrate	0.50 g
Sodium chloride	5.00 g
Sodium azide*	0.15 g
Bacto agar	15.00 g

Suspend 28.5g in 500mL DI in a 1L Erlenmeyer flask and boil to dissolve completely. Sterilize in autoclave for 15 minutes at 121°C. Cool medium to 45-50°C and aseptically dispense into 100 x 15mm petri plates. Final pH of the medium is 7.1 at 25°C.

***Note**: Sodium azide is potent neurotoxin. Avoid breathing dust. Avoid contact with eyes, skin, and clothing. Do not allow azide to come in contact with metal drain pipes. Flush with copious amounts of water when discarding down drains.*

Procedure

a) Shake sample well, 25-30 times, and measure 100mL in a sterile graduated cylinder. Add to a P-A culture bottle. Mix thoroughly to achieve adequate mixing of the concentrated medium and inoculum.

b) Incubate bottles for 24 hours at 35°C. The production of acid from the fermentation of the lactose turns the indicator yellow. Reincubate negative bottles for an additional 24 hours.

c) Transfer a sample of each positive culture to a tube of lauryl tryptose with MUG and azide dextrose broth using a sterile inoculating loop.

d) Incubate inoculated media for 24-48 hours at 35°C.

e) Gas production in lauryl tryptose broth with MUG is a presumptive positive test for total coliforms. Positive tubes are further examined under long wave (366nm) UV light for fluorescence which indicates the presence of glucuronidase positive E.coli. It is estimated that about 87% or greater of E.coli strains are B-D glucuronidase positive (Federal Register, 1991).

f) Azide dextrose broth tubes showing growth are streaked to bile esculin azide agar plates which are incubated for 24 hours at 35°C. Blackening

of the medium under the colonial growth confirms the presence of fecal streptococci.

Media Confirmation

Confirm performance of each new lot of medium using the following cultures:

Control Culture	Medium	Positive Reaction
ATCC 25922 *E. coli*	LTB+MUG	Gas formation + bright blue fluorescence under long wave UV illumination.
ATCC 23355 *E. aerogenes*	LTB+MUG	Gas formation, negative fluorescence under long wave UV illumination.
ATCC 29212 *S. fecalis*	Bile	Black halos surround colonial growth.
	Esculin Azide agar	
	Azide Dextrose broth	Abundant growth.

Data Analysis

a) Record growth as positive/negative for 100mL sample. Identify groups present and recommend subsequent sequential sampling of site.

Quality Control

a) Refer to general Quality Control section for a discussion of accepted QA/QC practices.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9221 E.

b) Federal Register, Environmental Protection Agency, 40 CFR Part 141 [WH-FRL-3871-2] National Drinking Water Regulations. Vol. 56, January 8, 1991.

c) Feng, P.C.S., and P.A. Hartman. "Fluorogenic Assays for Immediate Confirmation of *Escherichia coli*," Applied and Environmental Microbiology 43: 1320-1329. 1982.

d) Jacobs, N.J., et al. "Comparison of membrane filter, multiple tube fermentation tube, and presence-absence techniques for detecting total coliforms in small community water systems." Appl. Environ. Microbiol. 51:1007, 1986.

e) Monograph, Technical Information. "Bacto Lauryl Tryptose Broth with MUG." Difco Laboratories, Detroit, MI, 1986.

Revision History

February 14, 1994: Publication in the 1994 Lab Manual
November 14, 2002: SEAM codes replaced by EMS codes

Total Coliforms in Water by Multiple-Tube Fermentation (MTF) – Prescriptive

Parameter

Coliforms, Total

Analytical Method

Multiple Tube Fermentation

Introduction

This method is prescriptive. It describes the statistical estimation of total coliform density in environmental water sources such as fresh water, surface water, ground water, seawater, etc. This test can also be applied to wastewater and effluent samples.

Drinking water testing in BC must be performed by test methods approved by the BC Enhanced Water Quality Assurance (EWQA) Program, in compliance with the BC Drinking Water Protection Act. This method does not meet all EWQA requirements for drinking water testing, and does not define regulatory requirements for the analysis of drinking water samples originating in BC.

A licence must be obtained from the Public Health Agency of Canada (PHAC) to purchase the control organisms required for this test. Refer to the PHAC website.

Method Summary

The coliform group consists of several genera of bacteria belonging to the family Enterobacteriaccae. The historical definition of this group has been based on the method used for detection, lactose fermentation, rather than on the tenets of systematic bacteriology. Accordingly, when the fermentation technique is used, this group is defined as all facultative, anaerobic, gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 hours incubation at 35°C.

MDL(s) and EMS Method & Analyte Code(s)*

Analyte	Approx. MDL	EMS Analyte / Method Codes*
Total Coliforms	1 MPN /100 mL	0451 / X015
Total Coliforms, Confirmed	1 MPN /100 mL	0451 / 2495

*Refer to EMS Parameter Dictionary on the Ministry of Environment and Climate Change Strategy website for all current EMS codes.

Matrix

Water

Interferences and Precautions

The precision of the fermentation test in estimating coliform density depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examined shows acid and/or gas in some or all of the tubes and the smallest sample inoculum shows no acid and/or gas in any or a majority of tubes.

Work aseptically to prevent contamination of lab personnel and the lab area, and to prevent cross-contamination between samples. Refer to the *Government of Canada Canadian Biosafety Standard* for more information.

Sample Handling and Preservation

The sample is collected in the field and submitted unfiltered in a sterilized bacteriology bottle containing sufficient sodium thiosulfate to neutralize up to 15 mg/L residual chlorine, or a minimum of 10 mg anhydrous / 120 mL container or 15 mg of the pentahydrate form. Sodium Thiosulfate is effective in neutralizing the bactericidal effect of chlorine, neutralizing residual halogens, and preventing continuation of bactericidal action during sample transit.

Holding Time: Incubation must begin within 30 hours of sample collection for results to be valid (APHA 9060B, 2013). Minimum volume required for analysis is 100 mL (APHA 9221A, 2014).

Storage: The sample should be kept cool (at <10°C) during transport and storage until analysis. Do not freeze samples (APHA 9060A, 2013).

Procedure

Refer to detailed instructions provided within the APHA reference method for guidance on the execution of this test:

APHA 9221 Multiple-Tube Fermentation Technique for Members of the Coliform Group.

The APHA guidance for this test is prescriptive and must be followed without modification.

Where subsampling occurs, be sure to homogenize the sample well prior to sub-sampling.

If dilutions are needed, do not dilute the sample in buffered water. The reagents are already buffered and excessive buffer compounds can adversely affect the growth of the target organisms.

Use the completed (confirmed) test on as a quality control measure on at least 10% of coliform-positive non-potable water samples on a seasonal basis to ensure false positive test results are not reported.

Incubation temperatures and times are important to prevent false positive and false negative reactions. Incubation instructions are provided by the media manufacturer and must be followed.

Quality Control

Summary of QC Requirements

QC Component	Minimum Frequency	Minimum Data Quality Objectives
Method Blank (MB)	One per batch (max 20 samples)	Less than reported DL
Lab Duplicates (DUP)	1 per batch (max 20 samples)[1]	± 65% RPD
Positive & Negative Controls	One each per day per incubator	Expected reaction to confirm proper operation of incubator and performance of the test.

B.C. EWQA Program QC requirements for drinking water testing are more stringent, requiring duplicate samples at a frequency of 1 in 10 samples.

If DQOs are not met, repeat testing or report qualified test results.

Method Blank: The method blank is 100 mL sterile water that proceeds through the same sample handling processes as test samples, (including sodium thiosulfate if used with test samples; recommend preparing Method Blank in a sample bottle).

Laboratory Duplicates: Sample duplicates are prepared when sufficient sample is received to subsample for laboratory duplicates. Homogenize the sample well prior to subsampling. Process both aliquots through the same sample handling processes as test samples.

Positive / Negative Controls: Two are recommended. Using both each day confirms that the test is performing as expected for all target and non-target organisms and that the incubator is operating as expected (reaches correct temperature at correct rate). Refer to APHA 9020 for more information.

Proofing of sample bottles, organisms, reagents, and supplies by lot is recommended to demonstrate sterility and performance prior to use. Refer to APHA 9020 for more information on recommended Quality Control practices for this test.

References

APHA 9221 (2014) Multiple-Tube Fermentation Technique for Members of the Coliform Group.

APHA 9060 (2013) Samples.

APHA 9020 (2015) Quality Control.

Revision History

Feb 14, 1994	Publication in 1994 Lab Manual.
Nov 14, 2002	SEAM codes replaced by EMS codes.
Dec 20, 2019	Updated to BC Lab Manual Prescriptive Method format. APHA 9221 was revised in 2013. APHA 9060 was revised in 2013. APHA 9020 was revised in 2015. Prescriptive nature of test is confirmed. QC section updated to include Method Blanks and Duplicate Samples. Changed sample storage temperature to <10°C as per APHA 9060 (2013).

Thermotolerant Coliforms in Water by Multiple-Tube Fermentation (MTF) – Prescriptive

Parameter

Coliforms, Thermotolerant

Analytical Method

Multiple Tube Fermentation

Introduction

This method is prescriptive. It describes the statistical estimation of total coliform density in environmental water sources such as fresh water, surface water, ground water, marine water, etc. This test can also be applied to wastewater and effluent samples.

Drinking water testing in BC must be performed by test methods approved by the BC Enhanced Water Quality Assurance (EWQA) Program, in compliance with the BC Drinking Water Protection Act. This method does not meet all EWQA requirements for drinking water testing, and does not define regulatory requirements for the analysis of drinking water samples originating in BC.

A licence must be obtained from the Public Health Agency of Canada (PHAC) to purchase the control organisms required for this test. Refer to the PHAC website.

Method Summary

Thermotolerant coliforms (those that ferment lactose to produce gas at 44.5 °C), are a subset of the coliform group and were traditionally called fecal coliforms. However, they have also been documented in organically rich waters or tropical climates in the absence of recent fecal contamination. Therefore, testing for E. coli, a specific indicator of fecal contamination, is recommended.

Current regulations may require that Thermotolerant (formerly fecal) coliforms be identified and enumerated. In the multiple-tube fermentation technique, this group of organisms is identified by their ability to ferment lactose to produce gas at 44.5°C.

MDL(s) and EMS Method & Analyte Code(s)*

Analyte	Approx. MDL	EMS Analyte / Method Codes*
Thermotolerant Coliforms	1 MPN/100 mL	0450 / MTFT
Thermotolerant Coliforms, Confirmed	1 MPN/100 mL	0450 / X015

*Refer to EMS Parameter Dictionary on the Ministry of Environment and Climate Change Strategy website for all current EMS codes.

Matrix

Water

Interferences and Precautions

The precision of the fermentation test in estimating coliform density depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examined shows acid and/or gas in some or all of the tubes and the smallest sample inoculum shows no acid and/or gas in any or a majority of tubes.

Work aseptically to prevent contamination of lab personnel and the lab area, and to prevent cross-contamination between samples. Refer to the *Government of Canada Canadian Biosafety Standard* for more information.

Sample Handling and Preservation

The sample is collected in the field and submitted unfiltered in a sterilized bacteriology bottle containing sufficient sodium thiosulfate to neutralize up to 15 mg/L residual chlorine, or a minimum of 10 mg anhydrous / 120 mL container or 15 mg of the pentahydrate form. Sodium Thiosulfate is effective in neutralizing the

bactericidal effect of chlorine, neutralizing residual halogens, and preventing continuation of bactericidal action during sample transit.

Holding Time: Incubation must begin within 30 hours of sample collection for results to be valid (APHA 9060B, 2013). Minimum volume required for analysis is 100 mL (APHA 9221A, 2014).

Storage: The sample should be kept cool (at <10°C) during transport and storage until analysis. Do not freeze samples (APHA 9060B, 2013).

Procedure

Refer to detailed instructions provided within the APHA reference method for guidance on the execution of this test:

APHA 9221 Multiple-Tube Fermentation Technique for Members of the Coliform Group, Method E, Thermotolerant (Fecal) Coliform Procedure.

The APHA guidance for this test is prescriptive and must be followed without modification.

Refer to APHA 9020 for guidance on quality control practice guidelines and for the use and handling instructions for control organisms.

Where subsampling occurs, be sure to homogenize the sample well prior to sub-sampling.

If dilutions are needed, do not dilute the sample in buffered water. The reagents are already buffered and excessive buffer compounds can adversely affect the growth of the target organisms.

Incubation temperatures and times are important to prevent false positive and false negative reactions. Incubation instructions are provided by the media manufacturer and must be followed.

Use the completed (confirmed) test as a quality control measure on at least 10% of coliform-positive non-potable water samples on a seasonal basis to ensure false positive test results are not reported.

For data analysis, refer to the MPN table provided in APHA 9221 C.

Quality Control

Summary of QC Requirements

QC Component	Minimum Frequency	Minimum Data Quality Objectives
Method Blank (MB)	One per batch (max 20 samples)	Less than reported DL
Lab Duplicates (DUP)	1 per batch (max 20 samples)[1]	± 65% RPD
Positive & Negative Controls	One each per day per incubator	Expected reaction to confirm proper operation of incubator and performance of the test.

B.C. EWQA Program QC requirements for drinking water testing are more stringent, requiring duplicate samples at a frequency of 1 in 10 samples.
If DQOs are not met, repeat testing or report qualified test results.

Method Blank: The method blank is 100 mL sterile water that proceeds through the same sample handling processes as test samples, (including sodium thiosulfate if used with test samples; recommend preparing Method Blank in a sample bottle).

Laboratory Duplicates: Sample duplicates are prepared when sufficient sample is received to subsample for laboratory duplicates. Homogenize the sample well prior to subsampling. Process both aliquots through the same sample handling processes as test samples.

Positive / Negative Controls: Two are recommended. Using both each day confirms that the test is performing as expected for all target and non-target organisms and that the incubator is operating as expected (reaches correct temperature at correct rate). Refer to APHA 9020 for more information.

Proofing of sample bottles, organisms, reagents, and supplies by lot is recommended to demonstrate sterility and performance prior to use. Refer to APHA 9020 for more information on recommended Quality Control practices for this test.

References

1. APHA 9221 (2014) Multiple-Tube Fermentation Technique for Members of the Coliform Group, Method E, Thermotolerant (Fecal) Coliform Procedure.
2. APHA 9060 (2013) Samples.
3. APHA 9020 (2015) Quality Control.

Revision History

Feb 14, 1994	Publication in 1994 Lab Manual.
Nov 14, 2002	SEAM codes replaced by EMS codes.
Dec 20, 2019	Updated to BC Lab Manual Prescriptive Method format. Prescriptive nature of test is confirmed. APHA 9221 was revised in 2014. APHA 9060 was revised in 2013. APHA 9020 was revised in 2015. QC Section updated to include Method Blanks and Duplicate Samples. Changed sample storage temperature to <10C as per APHA 9060 (2013).

Fecal Coliform Bacteria in Solids, Soil, and Sludge by Multiple-Tube Fermentation (MTF) Technique – (MPN)

Parameter

Coliform, fecal

Analytical Method and EMS Codes

Fecal Coliform, Confirmed MPN : **0450 X390**

Scope

This method describes a multiple tube fermentation technique which estimates the Most Probable Number (MPN) of fecal coliforms in solids, soil, and sludge. The MPN method for coliforms is not influenced by turbidity and applies to:

- drinking waters, raw and treated (chlorinated, U.V.)
- swimming pools
- non-drinking waters, raw water sources, marine water, wastewater, sewage effluent (treated and untreated)
- soil, sediments and sludge

Principle

The coliform group of bacteria is the principal indicator of suitability of a water for domestic, industrial, or other uses. Experience has established the significance of coliform group density as a criterion of the degree of pollution and thus of sanitary quality. Coliforms are Gram negative, non-spore-forming, oxidase negative rods able to ferment lactose within 24-48 hours incubation at 35°C. Fecal coliforms, a sub-group of total coliforms present in the gut and feces of warm blooded animals, include organisms defined by their ability to ferment lactose in a suitable culture medium at 44.5 ± 0.2°C. Fecal coliforms cannot live or reproduce outside the intestinal tracts of their animal hosts.

Sample Handling

The sample is collected in the field and submitted unfiltered and unpreserved in a sterilized water bacteriology bottle or a Whirl-Pak™ bag. Samples should be kept at 4°C until testing. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum sample weight required for analysis is 50 grams.

Detection Limit

2 MPN/gram

Interferences

None

Precision

There are no standard reference materials for fecal coliforms.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 35 ± 0.5°C temperature.
b) Water bath that is capable of maintaining a stable 44.5 ± 0.2°C temperature.
c) Sterile disposable serological pipettes, 1mL and 10mL.
d) Lauryl tryptose broth.
e) EC Medium.
f) 18mm test tubes with inverted Durham tubes.
g) 20mm test tubes with inverted Durham tubes.
h) Autoclave for steam sterilization of glassware and media.
i) Bunsen burner.
j) Platinum inoculation loops, 3mm diameter.
k) Microscope slides and microscope with oil immersion lens.
l) Buffered water dilution blanks.
m) Sterile Stomacher® bags.
n) Stomacher®.

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION:

Dissolve 34.0g of potassium dihydrogen phosphate (KH_2PO_4) in 500mL deionized water (DI). Adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM CHLORIDE SOLUTION:
Dissolve 38g magnesium chloride ($MgCl_2$) in 1L DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

c) BUFFERED DILUTION WATER:
Add 1.25mL stock PO_4 buffer solution and 5 mL stock $MgCl_2$ solution to a 1L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

Dilution blanks: 10mL in 20mm test tubes
100mL in milk dilution bottles
Autoclave 10 - 100mL volumes at 121°C for 15 minutes.

d) LAURYL TRYPTOSE BROTH: SINGLE STRENGTH

Tryptose	20.00 g
Lactose	5.00 g
Dipotassium hydrogen phosphate, K_2HPO_4	2.75 g
Potassium dihydrogen phosphate, KH_2PO_4	2.75 g
Sodium chloride, NaCl	5.00 g
Sodium lauryl sulfate	0.10 g
Distilled water	1.00 L

Add ingredients to distilled water, mix thoroughly and heat to dissolve. pH should be 6.8 ± 0.2 after sterilization. Before sterilization, dispense 10mL aliquots of medium into 18mm fermentation tubes with an inverted vial in each tube. Place stainless steel closures on tubes and sterilize in autoclave for 15 minutes at 121°C. Do not fully open the autoclave door until chamber temperature has dropped below 75°C to avoid trapping air in the inverted vials.

e) LAURYL TRYPTOSE BROTH - DOUBLE STRENGTH

See formula listing above, and use twice the weight of each chemical except water.

Suspend 71.2 g in 1L DI and warm slightly to dissolve completely. Dispense 10mL aliquots into 20mm test tubes with inverted vial in each tube. Place stainless steel closures on tubes and sterilize in autoclave for 15 minutes at 121°C. Do not fully open the autoclave until chamber temperature has dropped below 75°C to avoid trapping air in the inverted vials.

f) EC MEDIUM

Tryptose	20.0 g
Lactose	5.0 g
Bile salts #3	1.5 g
Dipotassium hydrogen phosphate K_2HPO_4	4.0 g
Potassium dihydrogen phosphate KH_2PO_4	1.5 g
Sodium chloride	5.0 g

Suspend ingredients in 1L DI water and warm to dissolve. Dispense 10mL aliquots into 20mm test tubes with inverted fermentation vial in each tube. Place stainless steel closures on tubes and sterilize in autoclave for 15 minutes. at 121°C. Do not fully open the autoclave door until chamber temperature has dropped below 75°C to avoid trapping air in the inverted vials. Final pH of the medium is 6.9 ±0.2 at 25°C.

Procedure

PRESUMPTIVE TEST FOR FECAL COLIFORMS

a) Arrange fermentation tubes of lauryl tryptose broth (LTB) in rows of five tubes each in a test tube rack. The number of rows and the sample volumes depend upon the quality and character of the solids tested. Most solid samples will require additional dilutions.

b) Remove hard particles such as the occasional rock from the sample. If sample is essentially rocky, hand mix rather than use a blender or Stomacher® bag. To prepare solid or semi-solid samples weigh the sample and add phosphate buffer or 0.1% peptone water to make 10^{-1} dilution. Prepare the appropriate decimal dilutions of the homogenized slurry as quickly as possible to minimize settling.

c) Use double strength lauryl tryptose broth tubes for the initial sample volume of 10mL per tube. Use single strength broth tubes for all subsequent sample volumes.

d) Incubate inoculated tubes at 35 ± 0.5°C. After 24 ± 2 hours shake each tube gently and examine it for gas production, and if no gas has formed, reincubate and re-examine at the end of 48 ± 3 hours. Record presence or absence of gas production and/or heavy growth. Submit all presumptive positive tubes showing any amount of gas or heavy growth to the confirmed test.

CONFIRMED PHASE FOR FECAL COLIFORMS

a) Gently swirl each presumptive tube showing gas or heavy growth. With a sterile inoculating loop, transfer a loopful of each positive culture to tubes of EC medium. Do not allow inoculated EC medium to remain longer than 30 minutes on the bench before placing in 44.5°C water bath. The level of water in the bath must be high enough to cover the depth of the medium in the tubes.

b) Incubate the inoculated EC tubes for 24 hours at 44.5 ± 0.2°C.

c) Gas production in an EC broth culture is considered a positive fecal coliform reaction. Only tubes which are positive in the EC medium within 24 hours are used in the calculation of fecal coliforms.

Data Analysis

ESTIMATION OF BACTERIAL DENSITY

a) Precision of Fermentation Tube Test

Unless a large number of sample portions are examined, the precision of the fermentation tube test is rather low. Exercise great caution when interpreting the sanitary significance of coliform results obtained from the use of a few tubes with each sample dilution, especially when the number of samples from a given sampling point is limited.

b) Computing and Recording of MPN

Refer to section 4.5 of the Microbiological Quality Assurance/Quality Control section of this manual.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9221.
b) "Microbiological Methods for Monitoring the Environment" US Environmental Protection Agency, 600/8 - 78 - 017, 1978.
c) McQuaker, N. A Laboratory Manual for the Chemical Analysis of Waters, Wastewaters, Sediments and Biological Materials. Part II. Supplement, B.C. Ministry of Environment, pp 73-77, 1989.

Revision History

February 14, 1994:	Publication in 1994 Lab Manual
November 14, 2002:	SEAM Codes replaced by EMS Codes

Thermotolerant Coliform Bacteria in Bivalve Molluscan Shellfish by Multiple-Tube Fermentation (MTF) – Prescriptive

Parameter

Coliforms, Thermotolerant (Fecal)

Analytical Method

Multiple Tube Fermentation (MTF)

Introduction

This method describes the detection of thermotolerant coliform bacteria, traditionally called fecal coliform bacteria, in bivalve molluscan shellfish. Diluted samples of blended shellfish are analyzed using the Multiple Tube Fermentation (MTF) method to estimate bacterial numbers. Test results are provided as Most Probable Number (MPN). The MTF method is applied to health-significant bacteria such as coliforms, thermotolerant coliforms, and fecal streptococci; however, other classes of organisms such as the sulfur, iron, and nitrogen bacteria can also be enumerated.

Bacteriological water quality standards based on total coliform and thermotolerant coliform levels, as determined by the MTF method, are presently in use for the classification of potable waters, shellfish growing areas and swimming and contact sport waters.

Bacteriological analysis of samples using the MTF method is not routinely applicable to field work due to the extensive media and equipment requirements. Sophisticated mobile facilities are required to carry out MPN work in the field.

This method is prescriptive, and must be followed as described.

Method Summary

The Multiple-Tube Fermentation (MTF) method estimates thermotolerant coliform densities in a sample by the pattern of growth and gas formation in inoculated tubes at various dilutions, with test results expressed as Most Probable Number (MPN). The MPN is calculated based on probability formulas which are dependent upon the dilution ratio and number of tubes per dilution.

Thermotolerant coliforms belong to the larger group of total coliforms, and all are members of the Family Enterobacteriaeceae. Thermotolerant coliform are Gram-negative, oxidase negative rods which ferment lactose at 44.5°C. Thermotolerant coliforms are often used as indicators of sewage contamination in fresh and marine waters, sediments and shellstock, etc., as they do not reproduce outside their normal habitat, which is the intestinal tract of warm blooded animals, and they are more abundant in feces than other coliforms or pathogenic bacteria. Thermotolerant coliform test results can be used to estimate E.coli densities, but the proportion of other thermotolerant coliforms present will vary depending on the sample source. In waters receiving effluent rich in carbohydrates, the test is much less specific for E. coli. In such waters, the incidence of thermotolerant Klebsiella is markedly increased.

MDL(s) and EMS Method & Analyte Code(s)*

Analyte	Approx. MDL	EMS Analyte / Method Codes*
Thermotolerant (Fecal) Coliforms	18 CFU /100 g	0450 / X390

*Refer to EMS Parameter Dictionary on the Ministry of Environment and Climate Change Strategy website for all current EMS codes.

Matrix

Bivalve Molluscan Shellfish

Interferences and Precautions

Work aseptically to prevent contamination of lab personnel and the lab area, and to prevent cross-contamination between samples. Refer to the *Government of Canada Canadian Biosafety Standard* for more information.

Sample Handling and Preservation

Shellstock samples can be collected in the field from aquaculture lease sites or wild harvesting areas. Size and number of shellstock will vary depending upon the species. Clams should be rinsed in clean marine water that may be found in the sampling area. Shellstock samples may be collected in 7 - 10 mil thick plastic bags.

Holding Time: Analyze samples within 24 hours of collection.

Storage: Keep samples cool (at <10°C) during transport and storage until analysis. Do not freeze samples (APHA 9060B, 2013).

Apparatus and Materials

a) 25 mL wide mouth serological pipettes.
b) 10 mL serological pipettes.
c) 1 mL serological pipettes.
d) Sterile applicator sticks or 5 mm inoculating loops.
e) Sterile shucking knives.
f) Sterile brushes.
g) Sterile blender jars.
h) Gloves (including cut resistant gloves).
i) Blender (recommended with timer).
j) Incubator capable of maintaining 35 ± 0.5°C.
k) Water bath capable of maintaining 44.5 ± 0.2°C.
l) 20mm test tubes with inverted Durham tubes.
m) 16mm test tubes with inverted Durham tubes.
n) Autoclave for steam sterilization.

Sterile buffered dilution water.

Reagents

a) STOCK PHOSPHATE (PO4) BUFFER SOLUTION:

Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500mL deionized water (DI). Adjust to pH 7.2 ± 0.5 with 1 N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile

0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM SULFATE SOLUTION:

Dissolve 50 g $MgSO_4 \cdot 7H_2O$ in distilled water and dilute to 1 litre.

c) BUFFERED DILUTION WATER (DILUENT):

Add 1.25 mL stock phosphate buffer solution and 5.0 mL magnesium sulfate solution to a 1 litre volumetric flask and dilute to volume with distilled water.

d) COLIFORM MPN MEDIUM:

Lauryl tryptose broth (LTB), Presumptive Test (Difco 0241). This medium is commercially available:

Tryptose	20.0 g
Lactose	5.00 g
K_2HPO_4	2.75 g
KH_2PO_4	2.75 g
NaCl	5.00 g
Sodium lauryl sulfate	0.10 g
Distilled water	1.00 L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. Add Durham tubes (gas vials) to tubes. Double strength broth is prepared by using

the same weights of ingredients as above and reducing distilled water to 500 mL. Dissolve and dispense 10 mL of medium per tube, both single and double strength. Tubes should be of sufficient capacity to contain 1 mL inoculum + 10 mL single strength broth or 10 mL inoculum + 10 mL double strength broth. The pH of the medium should be approximately 6.8 ± 0.2 after autoclave sterilization at 15 psi for 15 minutes.

e) EC MEDIUM:

Fecal Coliform Confirmation (Difco 0314). This medium is commercially available:

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Bile salts No.3	1.5 g
K_2HPO_4	4.0 g
KH_2PO_4	1.5 g
NaCl	5.0 g
Distilled water	1.0 L

Add Durham tubes (gas vials) to tubes. Heat all ingredients in distilled water to dissolve and dispense 5 mL medium into each tube. Close tubes with metal or heat-resistant plastic caps. The pH of the medium should be 6.9 ± 0.2 after autoclave sterilization at 15 psi for 15 minutes. Follow manufacturer's specifications and/or internal laboratory procedures for media preparation and storage.

Procedure

a) Scrub gloves (heavy rubber, mesh etc.) with soap and water.

b) Discard shellfish with badly broken shells or those that are dead as evidenced by gaping shells. Scrape extraneous material from the shell using a sterile scrub brush, paying attention to crevices at shell junctions. Place cleaned shellstock in a clean container or on clean towels.

c) Prior to shucking, sterilize bench or other suitable working area with 70% alcohol. In addition re-sterilize gloves with 70% alcohol and then rinse with potable water.

d) Shuck 10 shellstock from the bill (not the hinge), transferring meat and liquor into a tared blender jar. Weigh the meats and shell liquor and add an equal weight of diluent (buffered water or 0.1% peptone water). Blend for 90 seconds and dilute to 1:10 by promptly adding 20g of the homogenate to 80mL of diluent.

e) When the shucked quantity from 10 specimens greatly exceeds 200g, and when the consistency of the sample permits, grind undiluted for 30 seconds, then transfer 200g of this preliminary grind to a second sterile blender jar, add an equal weight of diluent and proceed as outlined above.

f) When 10 shellfish yield a quantity of shucked material much less than 200g, make a 1:10 dilution directly in the blender jar by adding 90mL of diluent for every 10g of sample. Blend for 90 seconds.

g) When the consistency of a 1:2 dilution would result in a mixture too thick for effective blending, use 100 g of shucked meats and add 300mL of diluent. Blend for 2 minutes and transfer 40g of the ground material to 60mL of diluent.

h) When specimens are too large, and only a part of the animal is used for food, use only the edible portion for analysis; 100 - 200g of the sample is then blended as outlined in #6 above.

Note: *Prompt transfers will ensure that the blended sample does not separate out in the blender jar. Wide mouth pipettes are convenient for these transfers.*

i) Set up test tube racks with a sequence of test tubes which includes 5 or 10

replicate tubes per sample volume and at least 3 dilutions. If larger coliform numbers are expected, further serial dilutions should be made.

j) The 1:10 dilution should be shaken 30 times prior to the inoculation of a multiple tube series.

k) Use double strength lauryl tryptose broth (LTB) tubes for the initial sample volume of 10mL per tube. Use single strength lauryl tryptose broth tubes for subsequent sample volumes.

l) Inoculate each tube in a set of 5 or 10 with replicate sample volumes in increasing serial dilutions. Mix test portions in the medium by gentle agitation.

m) Promptly incubate tubes at 35 ± 0.5°C for 24 ± 2 hours. After incubation, examine each tube for growth, gas and/or acidic reaction (shades of yellow color). Gas production or acidic reaction showing in the Durham tubes is regarded as a presumptive-positive result. Gently tap the cap of any test tubes showing turbidity but no gas production or acid production. Proceed with step o with 24 hour presumptive-positive LTB tubes. Re-incubate and re-examine negative tubes at the end of 48 ± 3 h.

n) Detection of an acidic reaction (yellow color) and/or gas in the tubes or bottles within 48 ± 3 h constitutes a presumptive-positive reaction. The absence of acidic reaction and/or gas formation at the end of 48 ± 3 h of incubation constitutes a negative test. Proceed with step o with 48 hour presumptive-positive LTB tubes.

o) Transfer an aliquot of each positive LTB tube using a sterile loop or transfer stick to tubes of EC medium. Gently shake tubes to ensure mixing of inoculum with medium. Place tubes in a circulating water bath at 44.5 ± 0.2°C and ensure the water level is higher than the level of the medium in the test tubes. Incubate the tubes for 24 ± 2 hours.

p) Positive thermotolerant coliform reaction are indicated by EC tubes showing turbidity and gas production in 24 ± 2 h or less. Failure to produce gas (with little or no growth) constitutes a negative reaction.

All positive EC tubes are used to calculate the MPN value. Use the most current MPN Index to determine fecal coliform levels.

Quality Control

Summary of QC Requirements		
QC Component	**Minimum Frequency**	**Minimum Data Quality Objectives**
Method Blank (MB)	One per batch (max 20 samples)	Less than reported DL
Lab Duplicates (DUP)	1 per batch (max 20 samples)	± 65% RPD
Positive & Negative Controls	One each per day per incubator	Expected reaction to confirm proper operation of incubator and performance of the test.
If DQOs are not met, repeat testing or report qualified test results.		

Positive & Negative Controls: A selected number of positive EC tubes may be streaked onto Levines Eosin Methylene Blue (EMB) agar plates. Typical colonies are discrete and nucleated with or without metallic sheen. Coloured colonies that may be coalescent and mucoid, with a weak sheen, may be coliforms. Additional testing may include re-inoculation of EC medium with a single colony and/or a biochemical test strip.

Proofing of sample bottles, organisms, reagents, and supplies by lot is recommended to demonstrate sterility and performance prior to use. Refer to APHA 9020 for more information on recommended Quality Control practices for this test.

References

1. McQuaker, N.R., A Laboratory Manual for the Chemical Analysis of Waters, Wastewaters, Sediments and Biological Materials, Part I, B.C. Ministry of Environment, 1976.
2. Greenberg, A.E. and Hunt, D.A. (eds). Laboratory Procedures for the Examination of Seawater and Shellfish. 5th edition. APHA, 1985.
3. APHA 9221 (2014) Multiple-Tube Fermentation Technique for Members of the Coliform Group.
4. APHA 9060 (2013) Samples.
5. APHA 9020 (2015) Quality Control.

Revision History

Feb 14, 1994	Publication in 1994 Lab Manual.
Nov 14, 2002	SEAM codes replaced by EMS codes.
Dec 20, 2019	Updated to BC Lab Manual Prescriptive Method format. Prescriptive nature of test is confirmed. QC Section updated to include Method Blanks and Duplicate Samples.

Total Coliforms in Water by Membrane Filtration and mEndo Media – Prescriptive

Parameter

Coliforms, Total

Analytical Method

Membrane Filtration

Introduction

This method is prescriptive. It describes the selective isolation of total coliforms from environmental water sources such as fresh water, surface water, ground water, and seawater. This test can also be applied to wastewater and effluent samples.

Drinking water testing in BC must be performed by test methods approved by the BC Enhanced Water Quality Assurance (EWQA) Program, in compliance with the BC Drinking Water Protection Act. This method does not meet all EWQA requirements for drinking water testing, and does not define regulatory requirements for the analysis of drinking water samples originating in BC.

A licence must be obtained from the Public Health Agency of Canada (PHAC) to purchase the control organisms required for this test. Refer to the PHAC website.

Method Summary

Coliform bacteria are defined as bacteria that develop red colonies with a metallic (golden-green) sheen within 24 hours at 35° ± 0.5 °C on an Endo-type medium containing lactose. Some members of the total coliform group also produce dark red, mucoid, or nucleated colonies without a metallic sheen. When verified, these are classified as atypical coliform colonies. When purified cultures of coliform bacteria are tested, they produce negative cytochrome oxidase and positive β-galactosidase test reactions. Generally, pink (non-mucoid), blue, white or colourless colonies lacking sheen on Endo media are considered non-coliforms by this technique.

MDL(s), EMS Method, & Analyte Code(s)*

Method Version / Analyte	Approx. MDL	EMS Analyte / Method Codes*
MF - Quantitative		
Total Coliforms	1 CFU /100 mL	0451 / X022
MF - Qualitative		
Total Coliforms	present or absent	0451 / not available

*Refer to EMS Parameter Dictionary on the Ministry of Environment and Climate Change Strategy website for all current EMS codes.

Matrix

Water

Interferences and Precautions

The MF technique is useful in monitoring drinking water and a variety of natural waters. However, the MF technique has limitations, particularly when testing waters with high turbidity or large numbers of non-coliform (background) bacteria. If heterotrophic bacteria interference is exhibited, for example, sample results may need to be invalidated and new samples collected.

The type and quality of membrane filter affects the size, colouration and number of colonies significantly.

Work aseptically to prevent contamination of lab personnel and the lab area, and to prevent cross-contamination between samples. Refer to the *Government of Canada Canadian Biosafety Standard* for more information.

Sample Handling and Preservation

The sample is collected in the field and submitted unfiltered in a sterilized bacteriology bottle containing sufficient sodium thiosulfate to neutralize up to 15 mg/L residual chlorine, or a minimum of 10 mg anhydrous / 120 mL container or 15 mg of the pentahydrate form. Sodium Thiosulfate is effective in neutralizing the bactericidal effect of chlorine, neutralizing residual halogens, and preventing continuation of bactericidal action during sample transit.

Holding Time: Incubation must begin within 30 hours of sample collection for results to be valid. Minimum volume required for analysis is 100 mL (APHA 9060A 2013).

Storage: The sample should be kept cool (at <10°C) during transport and storage until analysis. Do not freeze samples (APHA 9060B 2013).

Procedure

Refer to detailed instructions provided within the APHA reference method for guidance on the execution of this test:

APHA 9222 Membrane Filter Technique for Members of the Coliform Group, Method B, Standard Total Coliform Membrane Filter Procedure using Endo Media.

The APHA guidance for this test is prescriptive and must be followed without modification.

Refer to APHA 9020 for guidance on quality control practice guidelines and for the use and handling instructions for control organisms.

Incubation temperatures and times are important to prevent false positive and false negative reactions. Incubation instructions are provided by the media manufacturer and must be followed.

Where subsampling occurs, be sure to homogenize the sample well prior to sub-sampling.

For data analysis, refer to reading instructions and calculations in APHA 9222B.

Quality Control

Summary of QC Requirements		
QC Component	**Minimum Frequency**	**Minimum Data Quality Objectives**
Method Blank (MB)	One per batch (max 20 samples)	Less than reported DL or Absent for P/A tests
Lab Duplicates (DUP)	1 per batch (max 20 samples)[1]	± 65% RPD
Positive & Negative Controls	One each per day per incubator	Expected reaction to confirm proper operation of incubator and performance of the test.
B.C. EWQA Program QC requirements for drinking water testing are more stringent, requiring duplicate samples at a frequency of 1 in 10 samples. If DQOs are not met, repeat testing or report qualified test results.		

Method Blank: The method blank is 100 mL sterile water that proceeds through the same sample handling processes as test samples, (including sodium thiosulfate if used with test samples; recommend preparing Method Blank in a sample bottle).

Laboratory Duplicates: Sample duplicates are prepared when sufficient sample is received to subsample for laboratory duplicates. Homogenize the sample well prior to subsampling. Process both aliquots through the same sample handling processes as test samples.

Positive / Negative Controls: Two are recommended: a total coliform and a non-coliform. Using both each day confirms that the test is performing as expected for all target and non-target organisms and that the incubator is operating as expected

(reaches correct temperature at correct rate). Refer to APHA 9020 for more information.

Proofing of sample bottles, organisms, reagents, and supplies by lot is recommended to demonstrate sterility and performance prior to use. Refer to APHA 9020 for more information on recommended Quality Control practices for this test.

References

1. APHA 9222 (2015) Membrane Filter Techniques for Members of the Coliform Group, Method B, Standard Total Coliform Membrane Filter Procedure using Endo Media.
2. APHA 9060 (2013) Samples.
3. APHA 9020 (2015) Quality Control.

Revision History

Feb 14, 1994	Publication in 1994 Lab Manual
Nov 14, 2002	SEAM codes replaced by EMS codes
Dec 20, 2019	Updated to BC Lab Manual Prescriptive Method format. APHA 9222 was revised in 2015. APHA 9020 was revised in 2015. APHA 9060 was revised in 2013. Prescriptive nature of test is confirmed. QC Section updated to include Method Blanks and Duplicate Samples. Changed sample storage temperature to <10°C as per APHA 9060 (2013).

Total Coliforms and *E. coli* in Water by Membrane Filtration and Chromocult® Coliform Agar (CCA) – Prescriptive

Parameter

Coliforms [Total and *E.coli*]

Analytical Method

Membrane Filtration

Introduction

This method is prescriptive. It describes the selective isolation of total coliforms and E.coli from environmental water sources such as fresh water, surface water, ground water, etc. This test can also be applied to wastewater and effluent samples.

Drinking water testing in BC must be performed by test methods approved by the BC Enhanced Water Quality Assurance (EWQA) Program, in compliance with the BC Drinking Water Protection Act. This method does not meet all EWQA requirements for drinking water testing, and does not define regulatory requirements for the analysis of drinking water samples originating in BC.

A licence must be obtained from the Public Health Agency of Canada (PHAC) to purchase the control organisms required for this test. Refer to the PHAC website.

Method Summary

Chromocult® Coliform Agar is a selective and differential chromogenic culture medium for the microbiological analysis of water samples. Within 24 hours incubation at 36°± 2°C, this medium enables the simultaneous detection, differentiation and enumeration of E. coli and coliform bacteria.

Counting of coliform bacteria is based on the ability of ß-D-galactosidase, an enzyme which is characteristic of coliform bacteria, to cleave the substrate Salmon-GAL. The reaction results in salmon red colored coliform bacteria colonies.

Counting of E. coli is based on the cleavage of both the substrates X-glucuronide by ß-D-glucoronidase and Salmon-GAL by ß-D-galactosidase, an enzyme combination, which is characteristic of E. coli. In the presence of E. coli both substrates are cleaved, resulting in colonies that take on a dark blue to violet color as opposed to the salmon red of other coliform bacteria colonies. Non-coliform bacteria appear as colorless.

Occasionally on mEndo medium, typical sheen colonies may be produced by non-coliform organisms. Verification of typical and atypical colonies is required.

MDL(s) and EMS Method & Analyte Code(s)*

Method Version / Analyte	Approx. MDL	EMS Analyte / Method Codes*
MF - Quantitative		
Total Coliforms	1 CFU /100 mL	0451 / not available
E. coli	1 CFU /100 mL	0147 / not available
MF - Qualitative		
Total Coliforms	present or absent	0451 / not available
E. coli	present or absent	0147 / not available

*Refer to EMS Parameter Dictionary on the Ministry of Environment and Climate Change Strategy website for all current EMS codes.

Matrix

Water

Interferences and Precautions

The MF technique is useful in monitoring clean, non-turbid samples. However, the MF technique has limitations when testing waters with high turbidity or large numbers of non-coliform (background) bacteria. If heterotrophic bacteria interference is exhibited, for example, sample results may need to be invalidated and new samples collected.

The type and quality of membrane filter can significantly affect the size, colouration, and number of colonies produced.

Work aseptically to prevent contamination of lab personnel and the lab area, and to prevent cross-contamination between samples. Refer to the Government of Canada Canadian Biosafety Standard for more information.

Sample Handling and Preservation

The sample is collected in the field and submitted unfiltered in a sterilized bacteriology bottle containing sufficient sodium thiosulfate to neutralize up to 15 mg/L residual chlorine, or a minimum of 10 mg anhydrous / 120 mL container or 15 mg of the pentahydrate form. Sodium Thiosulfate is effective in neutralizing the bactericidal effect of chlorine, neutralizing residual halogens, and preventing continuation of bactericidal action during sample transit.

Holding Time: Incubation must begin within 30 hours of sample collection for results to be valid. Minimum volume required for analysis is 100 mL (APHA 9060A 2013).

Storage: The sample should be kept cool (at <10°C) during transport and storage until analysis. Do not freeze samples (APHA 9060B 2013).

Procedure

Refer to detailed instructions provided within the APHA reference method for guidance on the execution of this test:

APHA 9222 Membrane Filter Technique for Members of the Coliform Group, Method J, Simultaneous Detection of Total Coliform and E. coli by Dual-Chromogen Membrane Filter Procedure.

The APHA guidance for this test is prescriptive and must be followed without modification.

Chromocult® Coliform Agar (CCA) media must be purchased from a commercial vendor; it cannot be prepared from basic ingredients.

Follow sample size selection and filtering procedures in APHA 9222B.

Where subsampling occurs, be sure to homogenize the sample well prior to sub-sampling.

Refer to APHA 9020 for guidance on quality control testing practices for the evaluation and maintenance of equipment, media and organisms.

Incubation temperatures and times are important to prevent false positive and false negative reactions. Incubation instructions are provided by the media manufacturer and must be followed.

For data analysis, refer to reading instructions in APHA 9222J.

Quality Control

Summary of QC Requirements		
QC Component	**Minimum Frequency**	**Minimum Data Quality Objectives**
Method Blank (MB)	One per batch (max 20 samples)	Less than reported DL or Absent for P/A tests
Lab Duplicates (DUP)	1 per batch (max 20 samples)[1]	± 65% RPD
Positive & Negative Controls	One each per day per incubator	Expected reaction to confirm proper operation of incubator and performance of the test.
B.C. EWQA Program QC requirements for drinking water testing are more stringent, requiring duplicate samples at a frequency of 1 in 10 samples. If DQOs are not met, repeat testing or report qualified test results.		

Method Blank: The method blank is 100 mL sterile water that proceeds through the same sample handling processes as test samples, (including sodium thiosulfate if used with test samples; recommend preparing Method Blank in a sample bottle).

Laboratory Duplicates: Sample duplicates are prepared when sufficient sample is received to subsample for laboratory duplicates. Homogenize the sample well prior to subsampling. Process both aliquots through the same sample handling processes as test samples.

Positive / Negative Controls: Three are recommended: E. coli, a total coliform other than E. coli and a non-coliform. Using all three each day confirms that the test is performing as expected for all target and non-target organisms and that the incubator is operating as expected (reaches correct temperature at correct rate). Refer to APHA 9020 for more information.

Proofing of sample bottles, organisms, reagents and supplies by lot is recommended to demonstrate sterility and performance prior to use. Refer to APHA 9020 for more information on recommended Quality Control practices for this test.

References

1. APHA 9222 (2015) Membrane Filter Technique for Members of the Coliform Group, Method J, Simultaneous Detection of Total Coliform and *E. coli* by Dual-Chromogen Membrane Filter Procedure.
2. APHA 9060 (2013) Samples.
3. APHA 9020 (2015) Quality Control.
4. EMD Millipore technical data sheet, Chromocult Coliform Agar.

Revision History

Dec 20, 2019 First edition.

Fecal Coliforms in Fresh Water, Wastewater and Marine Water by Membrane Filter Technique (MF)

Parameter

Coliform, fecal

Analytical Method and EMS Codes

Membrane filter : **0450 X022**

Scope

This method describes the selective isolation of fecal coliforms from fresh water, wastewater, and marine water. Non-turbid water samples are passed through a 0.45µm membrane filter which is placed on a selective agar (m FC) for 18 - 24 hours incubation at 44.5°C for growth of fecal coliform colonies.

Principle

Fecal coliforms belong to the larger group of total coliforms, and all are members of the Family Enterobacteriaceae. Fecal coliforms are Gram-negative, oxidase negative, fermentative rods that will grow at 44.5°C. Fecal coliforms are chosen as indicators of fecal contamination of water supplies because they do not reproduce outside their normal habitat which is the intestinal tract of animals or humans. Numerous studies have shown positive correlations between the presence of fecal coliforms in water and the incidence of gastrointestinal disturbances or other pathology in people who drink or otherwise contact the water.

Sample Handling

The sample is collected in the field and submitted unfiltered and unpreserved in a sterilized water bacteriology bottle. Chlorinated water samples should be treated with sodium thiosulfate (final concentration 0.01% w/v) to neutralize the bactericidal effect of chlorine. The sample should be kept at 4°C until analysis. Variations in temperature are to be avoided. Minimum volume required for analysis is 125mL.

Range

0 - 100,000,000 CFU/100mL

Detection Limit

a) for duplicate 50mL samples the detection limit is 2 CFU/100mL.

b) for a total of 100mL the detection limit is 0 CFU/100mL.

Interferences

Excessive turbidity or particulate matter can interfere with filtration or cause clumping of the organism. Residual chlorine in chlorinated water must be neutralized with sodium thiosulfate at time of sampling.

Precision

Samples seeded with 20 and 200 CFU/100mL E. coli gave coefficients of variation of 11% and 9% respectively.

Apparatus and Materials

a) Heat sink incubator or water bath that is capable of maintaining a stable 44.5 ± 0.2°C temperature.
b) Sterile disposable serological pipettes, 1mL and 10mL.
c) Sterile 100mL glass graduated cylinders.
d) Sterile disposable petri dishes, 50mm x 12mm with tight fitting lids.
e) Sterile disposable petri dishes, 100mm x 15mm.
f) m-FC Agar.
g) BHI Agar.
h) Lauryl tryptose broth in 18mm test tubes with inverted fermentation vials (Durham tubes).
i) EC medium in 20mm test tubes with inverted Durham tubes.
j) Autoclave for steam sterilization of glassware and media.
k) Bunsen burner.

l) Platinum inoculation loops, 3mm diameter.

m) Glass filtration units, 250mL (Millipore® or equivalent), sterilized and wrapped in aluminum foil or kraft paper.

n) Presterilized membrane filters, 47mm diameter, white, grid marked, 0.45µm pore size, certified for bacteriology.

o) Vacuum source.

p) Vacuum flask and manifold to hold filtration units.

q) Smooth tipped forceps.

r) 95% ethanol, not denatured.

s) Microscope slides and microscope with oil immersion lens.

t) API 20 E® strips (available commercially from Analytab Products).

u) Oxidase reagent in sealed glass ampules (available from Difco or equivalent.)

v) Stereobinocular microscope with cool white fluorescent light source.

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION.

Dissolve 34.0g of potassium dihydrogen phosphate (KH_2PO_4) in 500mL deionized water (DI). Adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM CHLORIDE SOLUTION.

Dissolve 38g magnesium chloride ($MgCl_2$) in 1L DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

c) BUFFERED DILUTION WATER.

Add 1.25mL stock PO_4 buffer solution and 5mL stock $MgCl_2$ solution to a 1L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

Dilution blanks :	10mL in 20mm test tubes
	100mL in milk dilution bottles
Rinse water:	1500mL per 2L Erlenmeyer flask

Autoclave 10 - 100mL volumes at 121°C for 15 minutes; for larger volumes, increase the time as appropriate to achieve sterilization.

d) m-FC AGAR (DIFCO)

1) Formula (grams per litre):

Bacto tryptose	10.0 g
Proteose peptone No. 3	5.0 g
Bacto yeast extract	3.0 g
Lactose	12.5 g
Bacto bile salts #3	1.5 g
Sodium chloride	5.0 g
Bacto agar	15.0 g
Aniline blue	0.1 g

Suspend all ingredients in 1L DI and heat to boiling. Boil for 1 minute to completely dissolve the powder.

2) Rosolic Acid Solution

Rosolic acid	0.1 g
0.2N NaOH	10.0 mL

Dissolve rosolic acid in 0.2N NaOH immediately before use. Add 10mL rosolic acid to 100mL m-FC after medium has been boiled. Cool to 45-50°C and pour into sterile 50mm petri dishes (4 mL per plate). Final pH of medium is 7.4 at 25°C. Which may be stored for up to two weeks at 4°C.

e) LAURYL TRYPTOSE BROTH (DIFCO)

Bacto tryptose	20.0 g
Bacto lactose	5.0 g
Dipotassium hydrogen phosphate	2.75 g
Potassium dihydrogen phosphate	2.75 g
Sodium chloride	5.0 g
Sodium lauryl sulfate	0.1g

Suspend all ingredients in 1L DI and warm slightly to dissolve completely. Dispense 10mL aliquots into 18mm test tubes with inverted fermentation vial (Durham tube) in each tube. Place 18mm stainless steel closures on tubes and sterilize in autoclave for 15 minutes at 121°C. Do not fully open the autoclave door until chamber temperature has dropped below 75°C to avoid trapping air in the inverted vials. Final pH of the medium is 6.8 at 25°C.

f) EC MEDIUM (DIFCO)

Bacto tryptose	20.0 g
Bacto lactose	5.0 g
Bacto bile salts No.3	1.5 g
Dipotassium hydrogen phosphate	4.0 g
Monopotassium dihydrogen phosphate	1.5 g
Sodium chloride	5.0 g

Suspend all ingredients in 1L DI and warm slightly to dissolve completely. Dispense into 20mm test tubes with inverted fermentation vial (Durham tube) in each tube. Place 20mm stainless steel closures on tubes and sterilize in autoclave for 15 minutes at 121°C. Do not fully open autoclave door until chamber temperature has dropped below 75°C to avoid trapping air in the inverted vials. Final pH of the medium is 6.9 at 25°C.

g) BRAIN HEART INFUSION AGAR, dehydrated (DIFCO)

Formula (grams per litre)	
Calf brain infusion	200.0 g
Beef heart infusion	250.0 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Bacto agar	15.0 g

Suspend all ingredients in 1L DI and heat to dissolve completely. Sterilize in the autoclave for 15 minutes at 121°C. Cool to 45-50°C and aseptically dispense 15-17mL into 100mm petri dishes.

Procedure

a) Place a sterile membrane filter on a sterile filter base, grid side up, and attach the funnel to the base of the filter unit.

b) Select a sample volume to produce 20-80 colonies on the membrane filter. Decimal dilutions are prepared in 10mL buffered water dilution blanks. Do not filter less than 10mL volumes.

c) Shake the sample bottle vigorously about 30 times and measure the desired volume of the sample into a sterile graduated cylinder for volumes of 20mL or more, or pipet directly into the funnel for volumes of 10mL or decimal dilutions of 10mL.

d) Filter the sample and rinse the sides of the funnel with 20-30mL sterile buffered water. Turn off the vacuum and remove the funnel from the filter base.

e) Aseptically remove the membrane filter from the filter base and place grid side up on the m-FC agar. Reset if air bubbles are trapped under the filter.

f) Filter the samples in order of increasing sample concentration and/or volume, low bacterial density to high.

g) Prepare duplicate filters for each concentration or volume filtered. Incubate m-FC agar plates for 18 - 24 hours at 44.5°C.

h) Count blue or blue-grey colonies appearing after 18-20 hour incubation. For confirmation, colonies may be purified on BHIA and identified using an API® 20 E strip following the directions provided, or alternately, colonies may be transferred to lauryl tryptose broth, incubated at 35°C for 24-48 hours, and then confirmed in EC medium at 44.5°C for 24 hours.

i) Do not count pink or greenish colonies. Small blue colonies or excessively mucoid colonies should be confirmed.

Data Analysis

a) Calculate the bacterial density of Fecal Coliforms using the following formula:

$$(*\text{CFU}/100\text{mL}) = \frac{\text{Mean number of Fecal coliforms counted} \times 100}{\text{volume of sample filtered}}$$

*Colony forming units

b) Counts on plates with less than 20 colonies are noted as "estimated" counts.

c) Plates with no colonies are reported as less than the calculated value/100mL based on the single largest volume filtered. Values are routinely reported as <2 CFU/100mL for largest single volume of 50mL. However, if there are no colonies on either of the 50mL duplicate plates the volume may be taken as 100mL and reported as 0 CFU/100mL.

Quality Control

95% confidence limits for membrane plate counts are calculated as follows:

Counts between 1- 10			Counts between 11 - 20		
Counts	**Lower**	**Upper**	**Counts**	**Lower**	**Upper**
1	0.0	3.7	11	5.4	19.7
2	0.025	5.6	12	6.2	21.0
3	0.24	7.2	13	6.9	22.3
4	1.1	10.2	14	7.7	23.5
5	1.6	11.7	15	8.4	24.8
6	2.2	13.1	16	9.4	26.0
7	2.8	14.4	17	9.9	27.2
8	3.5	15.8	18	10.7	28.4
9	4.1	17.1	19	11.5	29.6
10	4.8	18.4	20	12.2	30.8

For counts greater than 20 use the following formulae:
upper limit = $C + 2\sqrt{C}$
lower limit = $C - 2\sqrt{C}$
Where C = number of colonies counted.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9222.
b) Dutka, B. Membrane Filtration: Applications, Techniques and Problems. Bernard Dutka (Ed.) Marcel Dekker, Inc. New York, 1981.
c) McQuaker, N. A Laboratory Manual for the Chemical Analysis of Waters, Wastewaters, Sediments and Biological Materials. Part II. Supplement, B.C. Ministry of Environment pp. 66-72, 1989.

Revision History

February 14, 1994: Publication in 1994 Lab Manual
November 14, 2002: SEAM codes replaced by EMS Codes

Escherichia Coli in Fresh and Marine Water by Membrane Filtration

Parameter

E. Coli

Analytical Method and EMS Code

Membrane filter : **0147 X387**

Scope

This method describes the culture of E. coli from fresh or marine water, using a pre-incubation step to recover stressed organisms. This method is unsuitable for water heavily contaminated with other saprophytic bacteria or for woodwaste leachate. Non-turbid water samples are passed through a 0.45µm membrane filter which is placed on mTEC agar for 2 hours incubation at 35°C followed by 22 hours incubation at 44.5°C for growth of fecal coliform colonies. E.coli are further confirmed by their inability to hydrolyze urea.

Principle

Escherichia coli make up the majority of bacteria grouped as fecal coliforms and are members of the Family Enterobacteriaceae. E. coli are Gram negative, oxidase negative, fermentative rods which grow and produce gas from lactose at 44.5°C. E. coli are chosen as indicators of fecal contamination of water supplies because they do not reproduce outside their normal habitat which is the intestinal tract of animals or humans. Numerous studies have shown positive correlations between the presence of E. coli in water and the incidence of gastrointestinal disturbances or other pathology in people who drink or otherwise contact the water. In low nutrient waters E. coli can be stressed and have an artificially low recovery rate on highly selective media.

Sample Handling

The sample is collected in the field and submitted unfiltered and unpreserved in a sterilized water bacteriology bottle. Chlorinated water samples should be treated with sodium thiosulfate (final concentration 0.01% w/v) to neutralize the bactericidal effect of chlorine. The sample should be kept at 4°C until analysis. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum volume required for analysis is 125mL.

Detection Limit

a) for 50mL samples the detection limit is 2 CFU/100mL.
b) for a total of 100mL the detection limit is 1 CFU/100mL.

Interferences

Excessive turbidity or particulate matter can interfere with filtration or cause clumping of the organism. Heavy growth of background bacteria, especially urease positive organisms, can obscure the recognition of E. coli. Residual chlorine in chlorinated water must be neutralized with sodium thiosulfate at the time of sampling.

Precision

There are no standard reference materials for E. coli. American Type Culture Collection (ATCC 25922) E. coli may be used to test performance and recovery on mTEC agar.

Apparatus and Materials

a) Heat sink incubator or water bath that is capable of maintaining a stable 44.5°C ± 0.2 temperature.
b) Incubator capable of maintaining a stable 35 ± 0.2 °C temperature, or programmable incubator with temperature programming to increase temperature from 35°C to 44.5°C.
c) Sterile disposable serological pipettes, 1 mL and 10 mL.
d) Autoclavable 100mL or 50mL glass graduated cylinders wrapped in kraft paper or foil and sterilized.
e) Sterile disposable petri dishes, 50mm x 12mm with tight fitting lids.
f) Sterile disposable petri dishes, 100mm x 15mm.
g) mTEC Agar.
h) Lauryl tryptose broth with MUG or lauryl sulfate broth with MUG.
i) EC medium or EC broth.
j) Non-fluorescing 18mm glass culture tubes with stainless steel closures and inverted fermentation vials or Durham tubes.
k) Brain heart infusion agar [BHIA].
l) Autoclave for steam sterilization of glassware and media and/or ultraviolet (UV) light source for disinfection of filter units.
m) Bunsen burner.
n) Platinum inoculation loops, 3mm diameter.
o) 250mL glass filtration units (Millipore® or equivalent), wrapped in aluminum foil or kraft paper and sterilized.
p) Presterilized membrane filters, 47mm diameter, white, grid marked, 0.45µm pore size, certified for bacteriology.
q) Vacuum source.
r) Vacuum flask and manifold to hold filtration units.
s) Smooth tipped forceps.
t) 95% ethanol.
u) Gram staining reagents.
v) Microscope slides and microscope with oil immersion lens.
w) API 20E® strips (Analytab Products Inc.).
x) Oxidase reagent in sealed glass ampules (Difco® or equivalent.)
y) Stereobinocular microscope with cool white fluorescent light source.
z) Long wave (366 nm) UV source.

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION.

Dissolve 34.0g of potassium dihydrogen phosphate (KH_2PO_4) in 500mL distilled or deionized water (DI). Adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM CHLORIDE SOLUTION.

Dissolve 38g magnesium chloride ($MgCl_2$) in 1L DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

c) BUFFERED DILUTION WATER.

Add 1.25mL stock PO_4 buffer solution and 5mL stock $MgCl_2$ solution to a 1L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

Dilution blanks: 10mL in 20mm test tubes
90mL in milk dilution bottles
Rinse water: 1500mL per 2L Erlenmeyer flask
Autoclave 10 - 100mL volumes at 121°C for 15 minutes; for larger volumes, increase the time as appropriate to achieve sterilization (follow recommendations of autoclave manufacturer).

d) BUFFERED DILUTION WATER - ALTERNATIVE (USEPA)

Sodium dihydrogen phosphate (NaH_2PO_4)	0.58 g
Sodium monohydrogen phosphate (Na_2HPO_4)	2.50 g
Sodium chloride (NaCl)	8.50 g

Dissolve the ingredients in 1L DI and dispense in appropriate amounts as listed above for dilution blanks. Autoclave at 121°C for 15 minutes. Final pH of the buffer should be 7.4 ± 0.2.

e) mTEC AGAR (DIFCO)

Formula (grams per litre):	
Proteose peptone #3	5.00 g
Bacto yeast extract	3.00 g
Lactose	10.0 g
Sodium chloride	7.50 g
Potassium dihydrogen phosphate KH_2PO_4	1.00 g
Dipotassium hydrogen phosphate K_2HPO_4	3.30 g
Sodium lauryl sulfate	0.20 g
Sodium desoxycholate	0.10 g
Bromcresol purple	0.08 g
Bromphenol red	0.08 g
Bacto agar	15.0 g

Suspend 22.65g powdered medium in 500mL DI in a 1L Erlenmeyer flask and heat to dissolve. Autoclave for 15 minutes at 121°C. Cool to 45 - 50°C and dispense 4mL per 50mm petri dish. Store at 4°C for up to 1 month. Final pH should be 7.3 at 25°C.

f) UREA SUBSTRATE

Urea	2.00 g
Phenol Red	0.01 g
DI water	100.00 mL

Grind urea and phenol red together in mortar with pestle. Add DI and stir to dissolve. Adjust solution pH to 5.0 ± 0.2 with 0.1 N NaOH. At this pH the solution is a straw-yellow colour. Do not autoclave. The solution may be stored at 4°C for up to one week.

g) LAURYL TRYPTOSE BROTH WITH MUG (DIFCO)

Formula (grams per litre):	
Bacto tryptose	20.00 g
Lactose	5.00 g

Dipotassium hydrogen phosphate K_2HPO_4	2.75 g
Potassium dihydrogen phosphate KH_2PO_4	2.75 g
Sodium chloride	5.00 g
Sodium lauryl sulfate	0.10 g
MUG (4-methylumbelliferyl-B-D-glucuronide)	0.05 g

Suspend 35.7 g powdered medium in 1L DI. Heat to dissolve completely. Dispense 10mL aliquots into 18mm test tubes with inverted Durham tubes. Autoclave for 15 minutes at 121°C. Allow autoclave temperature to drop below 75°C before opening door to avoid trapping air bubbles in the inverted vials. Final pH of the medium is 6.8 at 25°C.

h) BRAIN HEART INFUSION AGAR, dehydrated (DIFCO)

Formula (grams per litre):

Calf brain infusion	200.0 g
Beef heart infusion	250.0 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Agar	15.0 g

Suspend 26g in 500mL DI in a 1L Erlenmeyer flask and heat to boiling to dissolve completely. Sterilize in the autoclave for 15 minutes at 121°C. Cool to 45 - 50°C and aseptically dispense 15 -17mL aliquots into 100mm petri dishes. Final pH of medium is 7.4 at 25°C.

Procedure

a) Place a sterile membrane filter on a sterile filter base, grid side up and attach the funnel to the base of the filter unit.

b) Select a sample volume to produce 20-80 colonies on the membrane filter. Decimal dilutions are prepared in 90mL buffered water dilution blanks. Do not filter less than 10mL volumes.

c) Shake the sample bottle vigorously about 30 times and measure the desired volume of the sample into a sterile graduated cylinder for volumes of 20mL or more, or pipet directly into the funnel for volumes of 10mL or 10mL volumes of decimal dilutions.

d) Filter the sample and rinse the sides of the funnel with 20 - 30mL sterile buffered water. Turn off the vacuum and remove the funnel from the filter base.

e) Aseptically remove the membrane filter from the filter base and place grid side up on mTEC agar. Reset if air bubbles are trapped under the filter.

f) Filter the samples in order of increasing sample concentration and/or volume, low bacterial density to high.

g) Prepare duplicate filters for each concentration or volume filtered. Incubate mTEC agar plates for 2 hours at 35°C, followed by 20 ± 2 hours incubation at 44.5°C.

h) After incubation, remove filters from mTEC and reset on absorbent filter

pads saturated with 2mL urea substrate.

i) After 15-20 minutes at room temperature, count and record colonies remaining yellow to yellow-brown on filters. Colonies which have turned pink are urease positive.

j) For confirmation, colonies may be purified on BHIA, Gram stained and identified using an API 20E® strip following the directions provided. E. coli are oxidase negative, Gram negative rods (size: 1-1.5 µm x 2-3 µm).

k) Alternately, colonies may be picked to tubes of lauryl tryptose broth with MUG and incubated for 24-48 hours at 35°C. E. coli produces the enzyme glucuronidase which hydrolyzes MUG to yield a bright blue fluorescent product that is detectable under long wave (366 nm) UV light. ATCC 25922 E. coli may be cultured in parallel as a positive control; ATCC 13883 Klebsiella pneumoniae, as a negative control. It is estimated that about 87% or greater of E. coli strains are glucuronidase positive (Federal Register,1991). Tubes which do not fluoresce should be sub-cultured to EC medium and incubated in a 44.5°C waterbath for 24 hours for gas production.

l) Filters with heavy background growth will not be countable. Also, filters with heavy urease positive growth will obscure counts. This method is suitable for drinking water or recreational bathing waters only.

Data Analysis

a) Calculate the bacterial density using the following formula:

$$\text{E. coli(*CFU/100mL)} = \frac{\text{Mean number of E. coli counted}}{\text{Volume of sample filtered}} \times 100$$

*Colony forming units

b) Counts on plates with less than 20 colonies are noted as "estimated" counts.

c) Plates with no colonies are reported as less than the calculated value/100 mL based on the single largest volume filtered. Values are routinely reported as <2 CFU/100mL for largest single volume of 50mL. If there are no colonies on either of the 50mL duplicate plates the volume may be taken as 100mL and reported as <1CFU/100mL.

Quality Control

a) Refer to general quality control section for a discussion of accepted QA/QC practices.

b) From time to time positive sample plates should be read or reread by another analyst to confirm counts of typical colonies.

c) 95% confidence limits for membrane plate counts are calculated as follows:

Counts between 1 - 10			Counts between 11 - 20		
Counts	**Lower**	**Upper**	**Counts**	**Lower**	**Upper**
1	0.0	3.7	11	5.4	19.7
2	0.025	5.6	12	6.2	21.0
3	0.24	7.2	13	6.9	22.3
4	1.1	10.2	14	7.7	23.5
5	1.6	11.7	15	8.4	24.8
6	2.2	13.1	16	9.4	26.0
7	2.8	14.4	17	9.9	27.2
8	3.5	15.8	18	10.7	28.4
9	4.1	17.1	19	11.5	29.6
10	4.8	18.4	20	12.2	30.8

For counts greater than 20 use the following formulas:
upper limit = $C + 2\sqrt{C}$
lower limit = $C - 2\sqrt{C}$
Where C = number of colonies counted.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9213 D.
b) DuFour, A.P. Applied and Environmental Microbiology, 41: 1152. "Membrane Filter Method for Enumerating Escherichia coli," 1981.
c) Dutka, B. Membrane Filtration: Applications, Techniques and Problems. Bernard Dutka (Ed.) Marcel Dekker, Inc. New York, 1981.
d) Environmental Laboratory, Ministry of Environment and Parks. "E. coli Membrane Filter (MF) Analysis", pp. 1-10. (not dated)
e) Federal Register, Environmental Protection Agency, 40 CFR Part 141 [WH-FRL-3871-2] National Drinking Water Regulations.Vol. 56. January 8, 1991.
f) Monograph, Technical Information. "Bacto Lauryl Tryptose Broth with MUG." Difco Laboratories, Detroit, MI, 1986.

Revision History

February 14, 1994: Publication in 1994 Laboratory Manual
November 14, 2002: SEAM codes replaced by EMS Codes

Total Coliforms, Thermotolerant (Fecal) Coliforms and *E. coli* by Colilert® in Water – Prescriptive

Parameter Coliforms [Total, Thermotolerant (Fecal), and *E.coli*]

Analytical Method Enzyme Substrate Test

Introduction

This method is prescriptive. It describes the selective isolation of total coliforms, thermotolerant (fecal) coliforms, and *E. coli* from environmental water sources such as fresh water, surface water, ground water, etc. This test can also be applied to wastewater and effluent samples.

For marine water, Colilert-18 can be used for *E. coli* detection (but not for total coliforms) or Thermotolerant (fecal) coliforms).

Drinking water testing in BC must be performed by test methods approved by the BC Enhanced Water Quality Assurance (EWQA) Program, in compliance with the BC Drinking Water Protection Act. This method does not meet all EWQA requirements for drinking water testing, and does not define regulatory requirements for the analysis of drinking water samples originating in BC.

A licence must be obtained from the Public Health Agency of Canada (PHAC) to purchase the control organisms required for this test. Refer to the PHAC website.

Method Summary

The Colilert and Colilert-18 reagents simultaneously detect the presence of coliforms and *E. coli*. Two nutrient-indicators, ONPG and MUG, are metabolized by the coliform enzyme β-galactosidase and the *E. coli* enzyme β-glucuronidase, respectively. As coliform organisms grow during incubation at 35.0 ± 0.5°C, they use β-galactosidase to metabolize ONPG and change it from colourless to yellow. *E. coli* use β-glucuronidase to metabolize MUG and create fluorescence. Since most non-coliforms do not have these enzymes, they are unable to grow, and therefore they do not interfere or cause false positives. The few non-coliforms that do have these enzymes are selectively suppressed by the Colilert formulation.

Thermotolerant (fecal) coliforms are detected on a separate sample aliquot using the Colilert-18 hour reagent and an elevated incubation temperature of 44.5 ± 0.2°C, which is necessary to eliminate non-thermotolerant organisms.

The Presence/Absence tests for Total Coliforms and *E. coli* is performed directly in sample bottles. The Presence/Absence test for thermotolerant (fecal) coliforms is also performed directly in the same bottles, using a separate sample and the Colilert-18 hour reagent.

IDEXX Quanti-Tray and Quanti-Tray 2000 are semi-automated quantification methods based on the Most Probable Number (MPN) model as described in *Standard Methods for the Analysis of Water and Wastewater*. The 51–well tray is used for samples such as drinking and clean surface waters with an expected concentration range of the target organisms of 1 to 200 MPN / 100 mL. The 97-well tray has an auto-dilution feature that allows quantification from 1 to 2,419 MPN / 100 mL, and is suitable for clean water samples, effluents, wastewaters, or other samples where a higher count is expected.

Quantitative enzyme substrate tests for total coliforms, thermotolerant (fecal) coliforms, and *E. coli* from can also be performed in a multiple-tube format that results in a higher detection limit than the Quanti-Tray test. In the multiple-tube test a series of tubes are inoculated and incubated, and the resulting reaction is converted to MPN units. Refer to IDEXX for supplies and instructions.

MDL(s) and EMS Method & Analyte Code(s)*

Method Version / Analyte	Approx. MDL	EMS Analyte / Method Codes*
Multi-well/ Quanti-tray		
Total Coliforms	1 MPN /100 mL	0451 / X388
Thermotolerant (Fecal) Coliforms	1 MPN /100 mL	0450 / X388
E. coli	1 MPN /100 mL	0147 / X388
Presence / Absence		
Total Coliforms	present or absent	0451 / not available
Thermotolerant (Fecal) Coliforms	present or absent	0450 / not available
E. coli	present or absent	0147 / not available
Multiple-Tube Fermentation		
Total Coliforms	2 MPN / 100 mL	0451 / not available
Thermotolerant (Fecal) Coliforms	2 MPN / 100 mL	0450 / not available
E. coli	2 MPN / 100 mL	0147 / not available

*Refer to EMS Parameter Dictionary on the Ministry of Environment and Climate Change Strategy website for all current EMS codes.

Matrix

Water

Interferences and Precautions

Non-coliform bacteria, such as *Aeromonas*, *Flavobacterium*, and *Pseudomonas* species may produce small amounts of the β-D-galactosidase enzyme within the incubation time if present in concentrations of more than 10^6 CFU/100 mL.

Background colour in a water sample may interfere with interpretation of the colour produced after incubation. This interference is eliminated by comparing the colour produced by the sample to the colour of a control blank of the same sample, to which no reagent was added. Samples with a very high background colour must be diluted prior to analysis.

Excessive chlorine may interfere with this test. If a blue flash is seen when adding the Colilert reagent, the sample is considered to be invalid and the test must not be completed.

Colilert reagent is not intended to be used for samples altered by pre-enrichment or concentration, and therefore this test cannot be used as a confirmation step for cultures isolated by other tests.

Use on effluents that have been treated with brighteners or surfactants should be confirmed by running successive dilutions, as these products can produce fluorescence which can interfere with *E. coli* analysis.

Work aseptically to prevent contamination of lab personnel and the lab area, and to prevent cross-contamination between samples. Refer to the *Government of Canada Canadian Biosafety Standard* for more information.

Sample Handling and Preservation

The sample is collected in the field and submitted unfiltered in a sterilized bacteriology bottle containing sufficient sodium thiosulfate to neutralize up to 15 mg/L residual chlorine, or a minimum of 10 mg anhydrous / 120 mL container or 15 mg of the pentahydrate form. Sodium Thiosulfate is effective in neutralizing the bactericidal effect of chlorine, neutralizing residual halogens, and preventing continuation of bactericidal action during sample transit.

Holding Time: Incubation must begin within 30 hours of sample collection for results to be valid. Minimum volume required for analysis is 100 mL (APHA 9060A 2013).

Storage: The sample should be kept cool (at <10°C) during transport and storage until analysis. Do not freeze samples (APHA 9060B, 2013).

Procedure

Refer to detailed instructions provided by the Colilert® test method vendor (IDEXX) for guidance on the execution of these tests. Vendor guidance for this test is prescriptive and must be followed without modification.

Instructions for the quantitative, presence/absence, and multiple-tube tests accompany the supplies purchased from IDEXX, and are also available on their website.

Note that use and handling instructions for control organisms and quality control practice guidelines are not described in the vendor's instructions. Refer to APHA 9020 for guidance on these topics.

Where subsampling occurs, be sure to homogenize the sample well prior to sub-sampling.

Incubation temperatures and times are important to prevent false positive and false negative reactions. Incubation instructions are provided by the media manufacturer and must be followed.

If dilutions are needed, do not dilute the sample in buffered water. The reagents are already buffered and excessive buffer compounds can adversely affect the growth of the target organisms.

For data analysis, refer to the MPN table provided by IDEXX for the specific test performed.

Quality Control

Summary of QC Requirements		
QC Component	**Minimum Frequency**	**Minimum Data Quality Objectives**
Method Blank (MB)	One per batch (max 20 samples)	Less than reported DL or Absent for P/A tests
Lab Duplicates (DUP)	1 per batch (max 20 samples)[1]	± 65% RPD
Positive & Negative Controls	One each per day per incubator	Expected reaction to confirm proper operation of incubator and performance of the test.
B.C. EWQA Program QC requirements for drinking water testing are more stringent, requiring duplicate samples at a frequency of 1 in 10 samples. If DQOs are not met, repeat testing or report qualified test results.		

Method Blank: The method blank is 100 mL sterile water poured into a 120 mL sample bottle, (containing sodium thiosulfate if used with test samples) and proceeds through the same sample handling processes as test samples.

Laboratory Duplicates: Sample duplicates are prepared when sufficient sample is received to subsample for laboratory duplicates. Homogenize the sample well prior to subsampling into individual 120 mL sample bottles. Process both aliquots through the same sample handling processes as test samples.

Positive / Negative Controls: Three are recommended: *E. coli*, a total coliform other than *E. coli* and a non-coliform. Using all three each day confirms that the reagent is performing as expected for all target and non-target organisms and that

the incubator is operating as expected (reaches correct temperature at correct rate). Refer to APHA 9020 for more information.

Proofing of sample bottles, organisms, reagents, and supplies by lot is recommended to demonstrate sterility and performance prior to use. Refer to APHA 9020 for more information on recommended Quality Control practices for this test.

References

1. APHA 9223 (2016) Enzyme Substrate Coliform Test.
2. APHA 9060 (2013) Samples.
3. APHA 9020 (2015) Quality Control.
4. IDEXX Instructions. Prescriptive instructions available on the IDEXX website.

Revision History

Feb 14, 1994	Publication in 1994 Lab Manual
Nov 14, 2002	SEAM codes replaced by EMS codes
Nov 14, 2004	Formatting changes. Addition of Revision History section.
Dec 20, 2019	Revised format, updated references: APHA 9223 was revised 2016. APHA 9060 Samples was revised 2013, APHA 9020 was revised 2015. Confirmed that test is prescriptive with reference to detailed procedures on IDEXX website. Added Thermotolerant (Fecal) Coliform testing to method. Updated QC section to include Method Blanks and Duplicate Samples. Changed sample storage temperature to <10°C as per APHA 9060 (2013).

Heterotrophic Plate Count (HPC) in Water – Prescriptive

Parameter Heterotrophic Plate Count

Analytical Methods
Pour Plate
Spread Plate
Membrane Filtration
Enzyme Substrate

Introduction

The heterotrophic plate count (HPC), formerly known as the standard plate count, is a procedure for estimating the number of live culturable g-heterotrophic bacteria in water and measuring changes during water treatment and distribution or in swimming pools.

Four prescriptive test method options are described in the Method Summary section below and are authorized for use.

A licence must be obtained from the Public Health Agency of Canada (PHAC) to purchase the control organisms required for these tests. Refer to the PHAC website.

Method Summary

Four applicable prescriptive test method options may be used for HPC testing:

a) Pour Plate: The procedure is simple to perform and can accommodate volumes of sample or diluted sample ranging from 0.1 to 2.0 mL. The colonies produced are relatively small and compact, and less likely to encroach on each other than those produced by surface growth. However, submerged colonies can be slower growing and difficult to transfer. A thermostatically controlled water bath is essential for tempering the agar, and care is needed to prevent heat shocking the bacteria when dispensing the hot agar. Replicating every volume and dilution plated analyses is not required.

b) Spread Plate: This procedure causes no heat shock and all colonies are on the agar surface where they can be easily distinguished from particles and bubbles. Colonies can be quickly transferred and morphology easily discerned. However, this method is limited by the small volume of sample or diluted sample that can be absorbed by the agar: 0.1 to 0.5 mL depending on the degree to which the pre-poured plates have been dried. A supply of pre-dried, absorbent agar plates must be maintained to use this procedure.

c) Membrane Filtration: This procedure permits testing of large volumes of low-turbidity water. It produces no heat shock. Disadvantages include the expense for the membrane filtration equipment, the smaller display area of the filter, the need to detect colonies by reflected light against a white background if coloured filters or contrast stains are not used, possible damage to cells by excessive filtration pressures, and possible variations in membrane filter quality.

d) Enzyme Substrate: This procedure can be used with samples having a wide range of bacterial concentrations. The method uses a substrate-based medium in which the substrates are hydrolyzed by microbial enzymes causing the release of 4-methylumbelliferone maximally after 48 hours of incubation at 35°C. 4-Methylumbelliferone fluoresces when exposed to long-wavelength (365 nm) ultraviolet light. The number of fluorescing wells corresponds to a most probable number (MPN) of bacteria in the sample. This test produces no heat shock and is comparable in performance to the pour plate method.

MDL(s) and EMS Method & Analyte Code(s)*

Method Version / Analyte	Approx. MDL	EMS Analyte / Method Codes*
Pour Plate Heterotrophic Plate Count	1 CFU / mL	SPCN / not available
Spread Plate Heterotrophic Plate Count	1 CFU / mL	SPCN / not available
Membrane Filtration Heterotrophic Plate Count	1 CFU / mL	SPCN / X385
Enzyme Substrate Multi-well / Quanti-tray Heterotrophic Plate Count	1 CFU / mL	SPCN / not available

*Refer to EMS Parameter Dictionary on the Ministry of Environment and Climate Change Strategy website for all current EMS codes.

Matrix

Water

Interferences and Precautions

Work aseptically to prevent contamination of lab personnel and the lab area, and to prevent cross-contamination between samples. Refer to the *Government of Canada Canadian Biosafety Standard* for more information.

Refer to Method Summaries for interferences particular to each method option.

Sample Handling and Preservation

The sample is collected in the field and submitted unfiltered in a sterilized bacteriology bottle containing sufficient sodium thiosulfate to neutralize up to 15 mg/L residual chlorine, or a minimum of 10 mg anhydrous / 120 mL container or 15 mg of the pentahydrate form. Sodium Thiosulfate is effective in neutralizing the bactericidal effect of chlorine, neutralizing residual halogens, and preventing continuation of bactericidal action during sample transit.

Holding Time: Incubation must begin within 24 hours of sample collection for results to be valid (9215A, 2016). Minimum volume required for analysis is 100 mL (APHA 9060A 2013).

Storage: The sample should be kept cool (at <10°C) during transport and storage until analysis. Do not freeze samples (APHA 9060B 2013).

Procedure

Consult the following reference methods / instructions for detailed procedures for the four applicable test method options:

i) APHA 9215 Section A Introduction.

ii) APHA 9215 Section B Pour Plate Method.

iii) APHA 9215 Section C Spread Plate Method.

iv) APHA 9215 Section D Membrane Filter Method

v) Enzyme Substrate Multi-well / Quanti-tray Method: Refer to manufacturer's instructions.

The APHA guidance and the manufacturer's guidance for the enzyme substrate method are prescriptive and must be followed without modification.

Where subsampling occurs, be sure to homogenize the sample well prior to sub-sampling.

Incubation temperatures and times are important to prevent false positive and false negative reactions. Incubation instructions are provided by the media manufacturer and must be followed.

For data analysis, refer to reading instructions in the applicable reference section.

Quality Control

Summary of QC Requirements		
QC Component	**Minimum Frequency**	**Minimum Data Quality Objectives**
Method Blank (MB)	One per batch (max 20 samples)	Less than reported DL
Lab Duplicates (DUP)	1 per batch (max 20 samples)	± 65% RPD
Positive & Negative Controls	One each per day per incubator	Expected reaction to confirm proper operation of incubator and performance of the test.
If DQOs are not met, repeat testing or report qualified test results.		

Method Blank: The method blank is 100 mL sterile water that proceeds through the same sample handling processes as test samples, (including sodium thiosulfate if used with test samples; recommend preparing Method Blank in a sample bottle).

Laboratory Duplicates: Sample duplicates are prepared when sufficient sample is received to subsample for laboratory duplicates. Homogenize the sample well prior to subsampling. Process both aliquots through the same sample handling processes as test samples.

Positive Control: Any organism that will provide a positive reaction is suitable to demonstrate that the incubator is operating as expected (reaches correct temperature at correct rate). Enterobacter aerogenes has been shown to have good performance characteristics for this test. Refer to APHA 9020 for more information.

Proofing of sample bottles, organisms, reagents and supplies by lot is recommended to demonstrate sterility and performance prior to use. Refer to APHA 9020 for more information on recommended Quality Control practices for this test.

References

1. APHA 9215 (2015) Heterotrophic Plate Count.
2. APHA 9060 (2013) Samples.
3. APHA 9020 (2015) Quality Control.
4. Enzyme Substrate Multi-well / Quanti tray Manufacturer's Instructions.

Revision History

Feb 14, 1994	Publication in 1994 Lab Manual
Nov 14, 2002	SEAM codes replaced by EMS codes
Dec 20, 2019	Updated to BC Lab Manual Prescriptive Method format. Prescriptive nature of test is confirmed. APHA 9215 was revised in 2015. APHA 9060 was revised in 2013. APHA 9020 was revised in 2015. QC Section updated to include Method Blanks and Duplicate Samples. Changed sample storage temperature to <10°C as per APHA 9060 (2013).

Fecal Streptococci in Fresh Water, Wastewater, and Marine Water by Multiple Tube Technique (MPN)

Parameter

Streptococci, fecal

Analytical Method and EMS Code

MPN - confirmed : **0454 X389**

Scope

This method describes the probability estimation of the numbers of fecal streptococci from fresh water, wastewater, and marine water. This method is not influenced by turbidity as is membrane filtration. Broth culture is thought to yield higher bacterial recovery then membrane filtration, and thus it is better suited for the recovery of stressed organisms. The MPN index table has a built-in high bias, which may account for the difference in numbers between membrane filtration and MPN analyses.

Principle

Fecal streptococci are Gram positive, catalase negative, non-spore forming cocci belonging to the Family Deinococcaceae, and the genus Streptococcus. There are a number of species represented; S. fecalis, S. fecium, S. avium, S. bovis, S. gallinarum and, S. equinus, all of which belong to Lancefield's Group D. The larger group of fecal streptococci is further divided into the sub-group, enterococci. The enterococci are S. fecalis, S. avium, S. fecium, and S. gallinarum. The normal habitat of fecal streptococci is the gastrointestinal tract of animals and humans.

Sample Handling

The sample is collected in the field and submitted unfiltered and unpreserved in a sterilized water bacteriology bottle. Chlorinated water samples should be treated with sodium thiosulfate (final concentration 0.01% w/v) to neutralize the bactericidal effect of chlorine. The sample should be kept at 4°C until analysis. Variations in temperature are to be avoided. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum volume required for analysis is 75mL.

Range

0 - 100,000,000 MPN/100mL

Detection Limit

2 MPN/100mL

Interferences

Residual chlorine in chlorinated water must be neutralized with sodium thiosulfate at time of sampling.

Precision

There are no standard reference materials for fecal streptococci. Mean recovery of American Type Culture Collection (ATCC) 29212 S.fecalis in Azide Dextrose broth confirmed on Bile Esculin Azide agar is 128% at 859 CFU/100mL.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 35 ± 0.5°C temperature.
b) Sterile disposable serological pipettes, 1 mL and 10 mL.
c) Azide dextrose broth.
d) Bile esculin azide agar.
e) 18mm test tubes with stainless steel closures.
f) 20mm test tubes with stainless steel closures.

g) Autoclave for steam sterilization of glassware and media.
h) Bunsen burner.
i) Platinum inoculation loops, 3mm diameter.
j) Microscope slides and microscope with oil immersion lens.
k) Buffered water dilution blanks, 10mL in 20mm test tubes.
l) Petri dishes, sterile disposable, 100 x 15mm.

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION.

Dissolve 34.0g of potassium dihydrogen phosphate (KH_2PO_4) in 500mL deionized water (DI). Adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM CHLORIDE SOLUTION

Dissolve 38g magnesium chloride ($MgCl_2$) in 1L DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

c) BUFFERED DILUTION WATER.

Add 1.25mL stock PO_4 buffer solution and 5mL stock $MgCl_2$ solution to a 1L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

Dilution blanks: 10mL in 20mm test tubes
100mL in milk dilution bottles
Autoclave 10 - 100mL volumes at 121°C for 15 minutes.

d) AZIDE DEXTROSE BROTH (DIFCO) SINGLE STRENGTH

Formula (grams per litre):

Bacto beef extract	4.5 g
Bacto tryptose	15.0 g
Bacto dextrose	7.5 g
Sodium chloride	7.5 g
Sodium azide*	0.2 g

*__Note__: *Sodium azide is a potent neurotoxin. Avoid breathing dust. Avoid contact with eyes, skin, and clothing. Do not allow azide to come in contact with metal drain pipes. Flush with copious amounts of water when discarding down drains.*

Suspend 34.7g in 1L DI and warm slightly to dissolve completely. Dispense 10mL aliquots into 18mm test tubes. Autoclave for 15 minutes at 121°C. Final pH of the medium is 7.2 at 25°C.

e) AZIDE DEXTROSE BROTH - DOUBLE STRENGTH

See formula listing above.
Suspend 69.4g in 1L DI and warm slightly to dissolve completely. Dispense 10mL aliquots into 20mm test tubes. Place 20mm stainless steel closures on tubes and sterilize in autoclave for 15 minutes at 121°C.

***Note**: the use of 20mm test tubes. 18mm tubes cannot contain the final volume of sample plus medium.*

f) BILE ESCULIN AZIDE AGAR, dehydrated (DIFCO)

Formula (grams per litre):

Bacto beef extract	5.00 g
Proteose peptone No. 3	3.00 g
Bacto tryptone	17.00 g
Bacto oxgall	10.00 g
Bacto esculin	1.00 g
Ferric ammonium citrate	0.50 g
Sodium chloride	5.00 g
Sodium azide*	0.15 g
Bacto agar	15.00 g

Note: Sodium azide is a potent neurotoxin. Avoid breathing dust. Avoid contact with eyes, skin, and clothing. Do not allow azide to come in contact with metal drain pipes. Flush with copious amounts of water when discarding down drains.*

Suspend 57g in 1L DI and boil to dissolve completely. Sterilize in autoclave for 15 minutes at 121°C. Do not fully open autoclave door until chamber temperature has dropped below 75°C. Cool medium to 50°C and aseptically dispense into 100 x 15mm petri plates. Final pH of the medium is 7.1 at 25°C.

Procedure

a) Set up test tube rack with a sequence of test tubes which includes at least 5 replicate tubes per sample volume and 3 dilutions. Wastewater samples will require additional dilutions.

b) Use double strength azide dextrose broth tubes for the initial sample volume of 10mL per tube. Use single strength azide dextrose broth tubes for all subsequent sample volumes.

c) Shake the sample bottle vigorously about 30 times and measure the desired volume of the sample into the tubes of azide dextrose broth. Use 10mL buffered water blanks to make decimal dilutions of the sample for inoculation.

d) Incubate tubes for 24 hours at 35°C. Examine each tube for turbidity at the end of 24 hours. If no definite turbidity is present, reincubate and read again at the end of 48 hours.

e) Transfer a loopful of each positive culture to sections marked off on plates of bile esculin azide agar. Streak for isolated colonies. Incubate inverted agar plates at 35°C for 24 hours. Brownish-black colonies with brown halos confirm the presence of fecal streptococci.

f) Brownish-black colonies with brown halos may be transferred to tubes of brain heart infusion broth containing 6.5% NaCl. Growth in 6.5% NaCl broth at 45°C indicates that the colony belongs to the enterococcus group.

g) Only tubes which are positive on bile esculin azide agar within 24 hours are used for the calculation of fecal streptococci.

h) Refer to a standard MPN index (Section 4.6 of the Microbiological Quality Assurance/Quality Control section of this manual) for calculation of bacterial density. Note that a minimum of 3 dilutions are required for this calculation. Use the set of dilutions which includes the highest dilution with all positive tubes and the next two higher dilutions.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9230 A.

Revision History

February 14, 1994:	Publication in 1994 Laboratory manual.
November 14, 2002:	SEAM Codes replaced by EMS codes.

Fecal Streptococci in Solids, Soils and Sludge by Most Probable Number (MPN)

Parameter

Streptococci, fecal

Analytical Method And EMS codes

MPN, confirmed : **0454 X390**

Scope

This method describes the probability estimation of the number of fecal streptococci from solids, soils and sludge. This method is not influenced by turbidity, and is the only method which can estimate bacterial numbers in soil or sludge.

Principle

Fecal streptococci are Gram positive, catalase negative, non-spore forming cocci belonging to the Family Deinococcaceae, and the genus Streptococcus. There are a number of species represented; S. fecalis, S. fecium, S. avium, S. bovis, S. gallinarum and S. equinus all of which belong to Lancefield's Group D. The larger group of fecal streptococci is further divided into the sub group, enterococci. The enterococci are S. fecalis, S. avium, S. fecium, and S. gallinarum. The normal habitat of fecal streptococci is the gastrointestinal tract of animals and humans.

Sample Handling

The sample is collected in the field and submitted unpreserved in a sterilized water bacteriology bottle or Whirl-Pak™. The bag sample should be kept at 4°C until analysis. Variations in temperature are to be avoided. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum volume required for analysis is 50 grams.

Range

0 - 100,000,000 MPN/gram wet weight

Detection Limit

2 MPN/gram.

Interferences

None.

Precision

There are no standard reference materials for fecal streptococci. Mean recovery of American Type Culture Collection (ATCC) 29212 S.fecalis in Azide Dextrose broth confirmed on Bile Esculin Azide agar is 128% at 859 CFU/100mL.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 35 ± 0.2°C temperature.
b) Sterile disposable serological pipettes, 1mL and 10mL.
c) Azide dextrose broth.
d) Bile esculin azide agar.
e) 20mm test tubes with stainless steel closures.
f) 18mm test tubes with stainless steel closures.
g) Autoclave for steam sterilization of glassware and media.
h) Bunsen burner.
i) Platinum inoculation loops, 3mm diameter.
j) Gram staining reagents (available commercially from Difco).
k) Microscope slides and microscope with oil immersion lens.
l) Buffered water dilution blanks, 10mL in 20mm test tubes.

m) Buffered water in milk dilution bottles, 100mL.
n) Stomacher® or equivalent.
o) Sterile Stomacher® bags (available from Canlab.)
p) Petri dishes, sterile disposable, 100 x 15mm.

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION.

Dissolve 34.0g of potassium dihydrogen phosphate (KH_2P0_4) in 500mL deionized water (DI). Adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM CHLORIDE SOLUTION.

Dissolve 38g magnesium chloride ($MgCl_2$) in 1L DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

c) BUFFERED DILUTION WATER.

Add 1.25mL stock PO_4 buffer solution and 5mL stock $MgCl_2$ solution to a 1L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

Dilution blanks: 10mL in 20mm test tubes
100mL in milk dilution bottles
Autoclave 10 - 100mL volumes at 121°C for 15 minutes.

d) AZIDE DEXTROSE BROTH (DIFCO) SINGLE STRENGTH

Formula (grams per litre):

Bacto beef extract	4.5 g
Bacto tryptose	15.0 g
Bacto dextrose	7.5 g
Sodium chloride	7.5 g
Sodium azide*	0.2 g

***Note**: Sodium azide is a potent neurotoxin. Avoid breathing dust. Avoid contact with eyes, skin, and clothing. Do not allow azide to come in contact with metal drain pipes. Flush with copious amounts of water when discarding down drains.*

Suspend 34.7g in 1L DI and warm slightly to dissolve completely. Dispense 10mL aliquots into 18mm test tubes. Place 18mm stainless steel closures on tubes and sterilize in autoclave for 15 minutes at 121°C using the liquid. Final pH of the medium is 7.2 at 25°C.

d) AZIDE DEXTROSE BROTH - DOUBLE STRENGTH

See formula listing above.

Suspend 69.4g in 1L DI and warm slightly to dissolve completely. Dispense 10mL aliquots into 20mm test tubes. Place 20mm stainless steel closures on tubes and sterilize in autoclave for 15 minutes at 121°C.

Note: *the use of 20mm test tubes. 18mm tubes cannot contain the final volume of sample plus medium.*

f) BILE ESCULIN AZIDE AGAR, dehydrated (DIFCO)

Formula (grams per litre):

Bacto beef extract	5.00 g
Proteose peptone No. 3	3.00 g
Bacto tryptone	17.00 g
Bacto oxgall	10.00 g
Bacto esculin	1.00 g
Ferric ammonium citrate	0.50 g
Sodium chloride	5.00 g
Sodium azide*	0.15 g
Bacto agar	15.00 g

Note: Sodium azide is a potent neurotoxin. Avoid breathing dust. Avoid contact with eyes, skin, and clothing. Do not allow azide to be in contact with metal drain pipes. Flush with copious amounts of water when discarding down drains.*

Suspend 57g in 1L DI and heat to dissolve completely. Sterilize in autoclave for 15 minutes at 121°C. Do not fully open autoclave door until chamber temperature has dropped below 75°C. Cool medium to 50°C and aseptically dispense into 100 x 15mm petri plates. Final pH of the medium is 7.1 at 25°C.

Procedure

a) Set up test tube rack with a sequence of test tubes which includes at least 5 replicate tubes per sample volume and 3 dilutions. Most solid samples will require additional dilutions.

b) Use double strength azide dextrose broth tubes for the initial sample volume of 10mL per tube. Use single strength azide dextrose broth tubes for all subsequent sample volumes.

c) Weight 10g solid sample in sterile Stomacher® bag and add 100mL sterile buffered dilution water. Place original bag within additional bags if sample contains bits of vegetation, to strengthen the bag. Remove hard particles such as the occasional rock. If sample is essentially rocky, hand mix rather than stomach. Place bag containing sample and buffer in Stomacher® and stomach for about 30 seconds. Keep material in suspension while measuring the desired volume of the sample into the tubes of azide dextrose broth. Use 10mL buffered water blanks for decimal dilutions of sample for inoculation.

d) Incubate tubes for 24 hours at 35°C. Examine each tube for turbidity at the end of 24 hours. If no definite turbidity is present, reincubate and read again at the end of 48 hours.

e) Transfer a loopful of each positive culture to sections marked off on plates of bile esculin azide agar. Streak for isolated colonies. Incubate inverted agar plates at 35°C for 24 hours. Brownish-black colonies with brown halos confirm the presence of fecal streptococci.

f) Brownish-black colonies with brown halos may be transferred to tubes of brain heart infusion broth containing 6.5% NaCl. Growth in 6.5% NaCl broth at 45°C indicates that the colony belongs to the enterococcus group.

g) Only tubes which are positive on bile esculin azide agar within 24 hours are used for the calculation of fecal streptococci.

h) Refer to a standard MPN Index (see section 4.6 of the Microbiological Quality Assurance/Quality Control section of this manual) for calculation of bacterial density. Note that a minimum of 3 dilutions are required for this calculation. Use the set of dilutions which includes the highest dilution with all positive tubes and the next two higher dilutions.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9230 A.

Revision History

February 14, 1994: Publication in 1994 Laboratory manual.
November 14, 2002: SEAM codes replaced by EMS codes.

Fecal Streptococcus in Fresh Water, Wastewater, and Marine Water by Membrane Filter (MF) Technique

Parameter

Streptococci, fecal

Analytical Method and EMS Codes

Membrane filter : **0454 X022**

Scope

This method describes the selective isolation of fecal streptococci from fresh water, wastewater, and marine water. Non-turbid water samples are passed through a 0.45µm membrane filter which is placed on a selective medium (KF agar) for 48 hours incubation at 35°C for growth of fecal streptococcal colonies.

Principle

Fecal streptococci are Gram positive, catalase negative, non-spore forming cocci belonging to the Family Deinococcaceae, and the genus Streptococcus. There are a number of species represented, S. fecalis, S. fecium, S. avium, S. bovis, S. gallinarum and S. equinus all of which belong to Lancefield's Group D. The larger group of fecal streptococci are further divided into the sub group, enterococci. The enterococci are S. fecalis, S. aviums, S. fecium, and S. gallinarum. The normal habitat of fecal streptococci is the gastrointestinal tract of animals and humans. In general, the fecal streptococci include species which are thought to be abundant in animal and avian sources, and proposals have been made to examine the relationship of fecal streptococci to fecal coliforms in order to determine whether contamination is of animal or human origin. Fecal streptococci can survive longer than fecal coliforms in water, and so great care must be taken to interpret the results of such ratios. For the most part, one-time sampling of an unknown source will not provide enough information to make a definitive statement as to the origin of such enteric bacteria.

Sample Handling

The sample is collected in the field in a sterilized water bacteriology bottle and submitted unfiltered and unpreserved. Chlorinated water samples should be treated with sodium thiosulfate (final concentration 0.01% w/v) to neutralize the bactericidal effect of chlorine. The sample should be kept at 4°C until analysis. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum volume required for analysis is 125 mL.

Range

0 - 100,000,000 CFU/100mL

Detection Limit

a) for duplicate 50mL samples the detection limit is 2 CFU/100mL
b) for a total of 100mL the detection limit is 1 CFU/100mL

Interferences

Excessive turbidity or particulate matter can interfere with filtration or cause clumping of the organisms. Residual chlorine in chlorinated water must be neutralized with sodium thiosulfate at time of sampling.

Precision

There are no standard reference materials for fecal streptococci. Mean recoveries of American Type Culture Collection (ATCC) cultures of S. fecalis on KF Streptococcus agar are 105% at 113 CFU/100mL and 101% at 11.3 CFU/100mL, with coefficients of variation of 8.7% and 16% respectively.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 35°C ± 0.5°C temperature.
b) Sterile disposable serological pipettes, 1mL and 10mL.
c) Sterile 100mL or 50mL glass graduated cylinders.
d) Sterile disposable petri dishes, 50mm x 12mm with tight fitting lids.
e) Sterile disposable petri dishes, 100mm x 12mm.
f) KF streptococcus agar.
g) Brain heart infusion agar (BHIA).
h) Hydrogen peroxide 3%.
i) Autoclave for steam sterilization of glassware and media.
j) Bunsen burner.
k) Platinum inoculation loops, 3mm diameter.
l) 250mL glass filtration units (Millipore or equivalent), sterilized and wrapped in aluminum foil or kraft paper.
m) Presterilized membrane filters, 47mm diameter, white, grid marked, 0.45µm pore size, certified for bacteriology.
n) Vacuum source.
o) Vacuum flask and manifold to hold filtration units.
p) Smooth tipped forceps.
q) 95% ethanol, undenatured.
r) Gram staining reagents (available commercially from Difco).
s) Microscope slides and microscope with oil immersion lens.
t) Stereobinocular microscope with cool white fluorescent light source.

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION.

Dissolve 34.0g of potassium dihydrogen phosphate (KH_2PO_4) in 500mL deionized water (DI). Adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM CHLORIDE SOLUTION.

Dissolve 38g magnesium chloride ($MgCl_2$) in 1L DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle Store at 4°C. Discard if solution becomes cloudy.

c) BUFFERED DILUTION WATER.

Add 1.25mL stock PO_4 buffer solution and 5mL stock $MgCl_2$ solution to a 1L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

Dilution blanks:	10mL in 20mm test tubes
	100mL in milk dilution bottles
Rinse water:	1500mL per 2L Erlenmeyer flask

Autoclave 10 - 100mL volumes at 121°C for 15 minutes; for larger volumes, increase the time as appropriate to achieve sterilization.

d) KF STREPTOCOCCUS AGAR, dehydrated (DIFCO)

Formula (grams per litre):

a) Proteose Peptone No.3	10.000 g
Bacto yeast extract	10.000 g
Sodium chloride	5.000 g
Sodium glycerophosphate	10.000 g
Maltose	20.000 g
Lactose	1.000 g
Sodium azide*	0.400 g
Bacto brom cresol purple	0.015 g
Bacto agar	20.000 g

***Note**: Sodium azide is a potent neurotoxin. Avoid breathing dust. Avoid contact with eyes, skin, and clothing. Do not allow azide to come in contract with metal drain pipes. Flush with copious amounts of water when discarding down drains.*

Suspend 56.4g in 1L DI and heat to dissolve completely. Continue to boil for 5 minutes, watching that it does not boil over. DO NOT AUTOCLAVE. Cool to 50°C and add 1mL of 1% TTC per 100 mL KF agar. Do not reheat medium after TTC has been added. Dispense 4mL per 50mm sterile petri plate. Store plates at 4°C for up to 1 month.

e) Triphenyltetrazolium chloride (TTC)

Make a 1% solution by suspending 0.1g in 10mL sterile DI. Vortex to dissolve. **DO NOT HEAT**. Use immediately. Do not store solution.

f) BRAIN HEART INFUSION AGAR (BHIA), dehydrated (DIFCO)

Formula (grams per litre):

Calf brain infusion	200.0 g
Beef heart infusion	250.0 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Agar	15.0 g

Suspend 52g in 1L DI and heat to dissolve completely. Sterilize in the autoclave for 15 minutes at 121°C. Cool to 45-50°C and aseptically dispense 15-17mL portions into 100mm petri dishes.

Procedure

a) Place a sterile membrane filter on a sterile filter base, grid side up, and attach the funnel to the base of the filter unit.

b) Select a sample volume to produce 20 - 80 colonies on the membrane filter. Decimal dilutions are prepared in 10mL buffered water dilution blanks. Do not filter less than 10mL volumes.

c) Shake the sample bottle vigorously about 30 times and measure the desired volume of the sample into a sterile graduated cylinder for volumes of 20mL or more, or pipet directly into the funnel for volumes of 10mL or decimal dilutions of 10mL.

d) Filter the sample and rinse the sides of the funnel with 20 - 30mL sterile buffered water. Turn off the vacuum and remove the funnel from the filter base.

e) Aseptically remove the membrane filter from the filter base and place grid side up on KF streptococcus agar plate. Reset if air bubbles are trapped under the filter.

f) Filter the samples in order of increasing sample concentration and/or volume, low bacterial density to high.

g) Prepare duplicate filters for each concentration or volume filtered. Incubate KF streptococcus agar plates for 48 hours at 35 ± 0.5°C.

h) Count red and pink colonies appearing after 48 hours incubation. Do not count buff or white colonies. For confirmation, pick individual colonies and prepare smears as follows:

1) Emulsify single colony in drop of DI on microscope slide and allow to air dry.
2) Heat fix smear by briefly passing slide through bunsen flame. Slide should be warm to touch on the back of hand, but not hot enough to burn.
3) Rest slide on a staining rack suspended over a sink. Cover smear with Gram's Crystal Violet for 1 minute.
4) Wash crystal violet off and cover smear with Gram's Iodine for 1 minute.
5) Wash iodine off and briefly decolorize with Gram's acetone alcohol. A faint violet colour should still be present in wash.
6) Counterstain with Gram's safranine for 30 seconds to 1 minute.
7) Wash slide well and blot dry.
8) Examine slide with oil immersion microscope. Streptococcus sp. are Gram-positive, slightly lanceolate cocci, cleaving on one plane.

Continue confirmation by picking a colony and emulsifying in 3% H_2O_2. Presence of bubbles in H_2O_2 is positive for catalase activity. Streptococcus sp. are catalase negative. The major source of false positives on KF agar are Staphylococcus sp., which are also gram positive cocci, but which cleave in more than one plane and are catalase positive.

Data Analysis

a) Calculate the bacterial density of Fecal Streptococci using the following formula:

$$(*\text{CFU}/100\text{mL}) = \frac{\text{Mean number of fecal streptococci}}{\text{Volume of sample filtered}} \times 100$$

* colony forming units

b) Counts on plates with less than 20 colonies are noted as "estimated" counts.

c) Plates with no colonies are reported as less than the calculated value/100mL based on the single largest volume filtered. Values are routinely reported as <2 CFU/100mL for largest single volume of 50mL.

However, if there are no colonies on either of the 50mL duplicate plates the volume may be taken as 100mL and reported as 1 CFU/100mL.

Quality Control

95% confidence limits for membrane filtration plate counts are calculated as follows:

Counts between 1 - 10			Counts between 11 - 20		
Counts	**Lower**	**Upper**	**Counts**	**Lower**	**Upper**
1	0.0	3.7	11	5.4	19.7
2	0.025	5.6	12	6.2	21.0
3	0.24	7.2	13	6.9	22.3
4	1.1	10.2	14	7.7	23.5
5	1.6	11.7	15	8.4	24.8
6	2.2	13.1	16	9.4	26.0
7	2.8	14.4	17	9.9	27.2
8	3.5	15.8	18	10.7	28.4
9	4.1	17.1	19	11.5	29.6
10	4.8	18.4	20	12.2	30.8

For counts greater than 20 use the following formulae:

upper limit = $C + 2\sqrt{C}$
lower limit = $C - 2\sqrt{C}$
Where C = number of colonies counted.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9230.
b) Dutka, B. Membrane Filtration: Applications, Techniques and Problems. Bernard Dutka (Ed.) Marcel Dekker, Inc. New York, 1981.
c) Environmental Laboratory, Ministry of Environment and Parks. (unpublished)"Fecal Streptococci Membrane Filter (MF) Analysis." pp 1-8.

Revision History

February 14, 1994: Publication in 1994 Laboratory manual
November 14, 2002: SEAM codes replaced by EMS codes

Enterococci in Fresh Water, Wastewater, and Marine Water by Membrane Filter Technique (MF)

Parameter

Enterococci

Analytical Method and EMS Codes

Membrane filter : **0148 X022**

Scope

This method describes the selective isolation of enterococci from fresh water, wastewater, and marine water. Non-turbid water samples are passed through a 0.45 µm membrane filter which is placed on a selective medium (mE agar) for 48 hours incubation at 41°C for growth of enterococci. Colonies are confirmed as enterococci by positive esculin hydrolysis on esculin iron agar.

Principle

Enterococci are Gram-positive, catalase negative, non-spore-forming cocci belonging to the Family Deinococcaceae, and the genus Streptococcus. There are a number of species represented; S. fecalis, S. fecalis subsp. liquefaciens, S. fecalis subsp. zymogenes, S. faecium, S. gallinarium and S. avium, all of which belong to Lancefield's Group D. The normal habitat of enterococci is the gastrointestinal tract of animals and humans. The enterococci are used as bacterial indicators of fecal contamination of recreational surface waters. Studies of marine and fresh water bathing beaches show a relationship between swimming-related gastroenteritis and the quality of the bathing water. Enterococci tend to survive longer than fecal coliforms, particularly in transit and are well suited as indicator organisms. Water quality guidelines for recreational waters of 33 CFU/100 mL (fresh) or 35 CFU/ 100mL (marine) have been proposed by the USEPA (1986).

Sample Handling

Samples are collected in the field in a sterilized water bacteriology bottle and submitted unfiltered and unpreserved. Chlorinated water samples should be treated with sodium thiosulfate (final concentration 0.01% w/v) to neutralize the bactericidal effect of chlorine. The sample should be kept at 4°C until analysis. Analysis must begin within 24 hours of sample collection for results to be valid. Minimum volume required for analysis is 125mL.

Detection Limit

a) for 50mL samples the detection limit is 2 CFU/100mL.
b) for a total of 100mL the detection limit is 1 CFU/100mL.

Interferences

Excessive turbidity or particulate matter can interfere with filtration or cause clumping of the organisms. Residual chlorine in chlorinated water must be neutralized with sodium thiosulfate at time of sampling.

Precision

There are no standard reference materials for enterococci. American Type Culture Collection (ATCC) cultures of S. fecalis may be used to test performance and recovery on mE agar.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 41±0.5°C temperature.
b) Sterile disposable serological pipettes, 1 mL and 10mL.
c) 100mL or 50mL glass graduated cylinders, sterilized.
d) Sterile disposable petri dishes, 50mm x 12mm with tight fitting lids.
e) Sterile disposable petri dishes, 100mm x 15mm.
f) mE Agar, or M-E Agar Base.
g) Esculin Iron Agar [EIA].
h) Brain Heart Infusion Agar [BHIA].
i) Hydrogen peroxide 3% (U.S.P.).
j) Autoclave for steam sterilization of glassware and media and/or ultraviolet (UV) light source for disinfection of filter units.
k) Bunsen burner.
l) Platinum inoculation loops, 3mm diameter.
m) Glass filtration units, 250mL (Millipore® or equivalent), wrapped in kraft paper or aluminum foil and sterilized.
n) Presterilized membrane filters, 47mm diameter, white, grid marked, 0.45µm pore size, certified for bacteriology.
o) Vacuum source.
p) Vacuum flask and manifold to hold filtration units.
q) Smooth tipped forceps.
r) 95% ethanol, undenatured.
s) Gram staining reagents.
t) Microscope slides and microscope with oil immersion lens.
u) Stereobinocular microscope with cool white fluorescent light source.
v) Milk dilution bottles or sterile, disposable dilution blanks, 90mL and 99mL.
w) Glass test tubes, 20mm.
x) Stainless steel test tube closures.

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION.

Dissolve 34.0g of potassium dihydrogen phosphate (KH_2PO_4) in 500mL deionized water or distilled water (DI). Adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM CHLORIDE SOLUTION.

Dissolve 38g magnesium chloride ($MgCl_2$) in 1L DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

c) BUFFERED DILUTION WATER.

Add 1.25mL stock PO_4 buffer solution and 5mL stock $MgCl_2$ solution to a 1L volumetric flask and make to volume with DI. Dispense into appropriate containers as follows:

Dilution blanks:	10mL in 20mm test tubes
	90mL in milk dilution bottles
Rinse water:	1500mL per 2L Erlenmeyer flask

Autoclave 10-100mL volumes at 121°C for 15 minutes; for larger volumes, increase the time as appropriate to achieve sterilization (follow recommendations of autoclave manufacturer).

d) BUFFERED DILUTION WATER - ALTERNATIVE (USEPA)

Sodium dihydrogen phosphate (NaH_2PO_4)	0.58 g
Sodium monohydrogen phosphate (Na_2HPO_4)	2.50 g
Sodium chloride (NaCl)	8.50 g

Dissolve the ingredients in 1 L DI and dispense in appropriate amounts as listed above for dilution blanks. Autoclave at 121°C for 15 minutes. Final pH of buffer should be 7.4 ± 0.2.

e) mE AGAR (DIFCO®)

Note: Use commercial formulations when possible.

1) Triphenyl tetrazolium chloride [TTC]
Make a 1% solution by suspending 0.1g in 10mL sterile DI. Vortex to dissolve. DO NOT HEAT. Use immediately. Do not store solution.

2) Nalidixic Acid
Suspend 0.25g nalidixic acid in 5mL DI; add a few drops of 0.1N NaOH to dissolve the acid.

3) Formula (grams per litre):

Bacto yeast extract	30.0 g
Bacto peptone	10.0 g
Sodium chloride	15.0 g
Esculin	1.0 g
Actidione	0.05 g
Sodium azide*	0.15 g
Bacto agar	15.0 g

***Note**: Sodium azide is a potent neurotoxin. Avoid breathing dust. Avoid contact with eyes, skin, and clothing. Do not allow azide to come in contract with metal drain pipes. Flush with copious amounts of water when discarding down drains.*

Suspend 35.6g mE powdered medium in 500mL DI in a 1L Erlenmeyer flask and heat to boiling to dissolve completely. Autoclave medium for 15 minutes at121°C. Cool to 45 - 50°C and add 1.5mL 1% TTC and 0.5mL dissolved nalidixic acid per 100 mL mE agar (7.5 mL 1% TTC and 2.5 mL nalidixic acid per 500 mL mE agar). Do not reheat medium after TTC has been added. Dispense 4 mL medium per 50mm sterile petri plate. Store plates at 4°C for up to 1 month.

f) ESCULIN IRON AGAR [EIA] (DIFCO)

Formula (grams per litre):

Esculin	1.0 g
Ferric ammonium citrate	0.5 g
Bacto agar	15.0 g

Suspend 8.25g medium in 500mL DI in a 1L Erlenmeyer flask and heat to dissolve completely. Autoclave medium for 15 min at 121°C. Cool to 45 - 50°C and dispense 4mL per 50mm sterile petri plate. Store plates at 4°C for up to 1 month.

g) BRAIN HEART INFUSION AGAR [BHIA], dehydrated (DIFCO)

Formula (grams per litre):

Calf brain infusion	200.0 g
Beef heart infusion	250.0 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	2.5 g
Agar	15.0 g

Suspend 52g in 1L DI and boil to dissolve completely. Autoclave for 15 minutes at 121°C. Cool to 45-50°C and aseptically dispense 15-17mL into 100mm petri dishes.

Procedures

a) Place a sterile membrane filter on a sterile filter base, grid side up, and attach the funnel to the base of the filter unit.

b) Select a sample volume to produce 20-80 colonies on the membrane filter. Decimal dilutions are prepared in 90 mL buffered water dilution blanks. Do not filter less than 10 mL volumes.

c) Shake the sample bottle vigorously about 30 times and measure the desired volume of the sample into a sterile graduated cylinder for volumes of 20mL or more, or pipet directly into the funnel for volumes of 10mL or 10mL volumes of decimal dilutions.

d) Filter the sample and rinse the sides of the funnel with 20 - 30mL sterile buffered water. Turn off the vacuum and remove the funnel from the filter base.

e) Aseptically remove the membrane filter from the filter base and place grid side up on mE agar plate. Reset if air bubbles are trapped under the filter.

f) Filter the samples in order of increasing sample concentration and/or volume, low bacterial density to high.

g) Prepare duplicate filters for each concentration or volume filtered. Incubate mE agar plates for 48 hours at 41± 0.5°C.

h) Note red and pink colonies appearing after 48 hours incubation.

i) Lift membrane from surface of mE agar and reset on warmed esculin iron agar. Incubate for up to 20 min at 41°C. Count red or pink colonies that produce a black zone of hydrolysis on the EIA. Lift the filter to confirm positive hydrolysis. Do not count buff or white colonies.

j) For additional confirmation, purify individual colonies on BHIA, incubate and pick isolated colonies for smears as follows:

1) Emulsify single colony in drop of DI on microscope slide and allow to air dry.
2) Heat-fix smear by briefly passing slide through bunsen flame. Slide should be warm to the touch on back of hand, but not hot enough to burn.
3) Rest slide on a staining rack suspended over a sink or staining tray. Cover smear with Gram's crystal violet for 1 minute.
4) Wash crystal violet off and cover smear with Gram's iodine for 1 minute.
5) Wash iodine off and briefly decolorize with Gram's acetone alcohol. A faint violet colour should still be present in wash.
6) Counterstain with Gram's safranine for 30 seconds to 1 minute.
7) Wash slide well and blot dry.
8) Examine slide with oil immersion microscope. Streptococcus sp. are Gram-positive, slightly lanceolate cocci, cleaving in one plane.

Continue confirmation by picking a colony and emulsifying in 3% H_2O_2. Presence of bubbles in hydrogen peroxide is positive for catalase activity. Streptococcus sp. are catalase-negative.

Data Analysis

a) Calculate the bacterial density using the following formula:

$$\text{Enterococci(*CFU/100mL)} = \frac{\text{Mean number of enterococci X 100}}{\text{Volume of sample filtered}}$$

*Colony forming units

b) Counts on plates with less than 20 colonies are noted as "estimated" counts.

c) Plates with no colonies are reported as less than the calculated value/100mL based on the single largest volume filtered. Values are routinely reported as <2 CFU/100mL for largest single volume of 50mL. If there are no colonies on either of the 50 mL duplicate plates the volume may be taken as 100mL and reported as <1 CFU/100mL.

Quality Control

a) From time to time positive sample plates should be read or reread by another analyst to confirm counts of typical colonies.

b) 95% confidence limits for membrane filtration plate counts are calculated as follows:

Counts between 1 - 10			Counts between 11 - 20		
Counts	**Lower**	**Upper**	**Counts**	**Lower**	**Upper**
1	0.0	3.7	11	5.4	19.7
2	0.025	5.6	12	6.2	21.0
3	0.24	7.2	13	6.9	22.3
4	1.1	10.2	14	7.7	23.5
5	1.6	11.7	15	8.4	24.8
6	2.2	13.1	16	9.4	26.0
7	2.8	14.4	17	9.9	27.2
8	3.5	15.8	18	10.7	28.4
9	4.1	17.1	19	11.5	29.6
10	4.8	18.4	20	12.2	30.8

For counts greater than 20 use the following formulae:

upper limit = $C + 2\sqrt{C}$
lower limit = $C - 2\sqrt{C}$
Where C = number of colonies counted.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9230.
b) Dutka, B. Membrane Filtration Applications, Techniques and Problems. Bernard Dutka (Ed.) Marcel Dekker, Inc. New York, 1989.
c) USEPA. Ambient Water Quality Criteria for Bacteria - 1986. EPA-440/5-84-002, U.S. Environmental Protection Agency, Washington, D.C., 1986.
d) Environmental Laboratory, Ministry of Environment and Parks. (Unpublished) "Enterococci Membrane Filter (MF) Analysis." pp 1-9.

Revision History

February 14, 1994:	Publication in 1994 Laboratory Manual.
November 14, 2002:	SEAM Codes replaced by EMS codes.

Enterococci in Water by MF or Enzyme Substrate Method – Prescriptive

Parameter	Enterococci
Analytical Methods	Membrane Filtration Enzyme Substrate

Introduction

Enterococci are Gram-positive, catalase negative, non-spore-forming cocci. The normal habitat of enterococci is the gastrointestinal tract of animals and humans.

This document describes two methods for the selective isolation of enterococci from environmental water sources such as fresh water, surface water, groundwater, and marine water. This test can also be applied to wastewater and effluent samples. It is intended for the analysis of environmental test samples (including those that may potentially be used as drinking water sources), but it is not intended as a method to confirm suitability of drinking water for human consumption.

Two prescriptive test method options are described in the Method Summary section below.

Drinking water testing in BC must be performed by test methods approved by the BC Enhanced Water Quality Assurance (EWQA) Program, in compliance with the BC Drinking Water Protection Act. This method does not meet all EWQA requirements for drinking water testing and does not define regulatory requirements for the analysis of drinking water samples originating in BC.

A licence must be obtained from the Public Health Agency of Canada (PHAC) to purchase the control organisms required for this test. Refer to the PHAC website.

Method Summary

The following two prescriptive test methods are authorized for use, and are described in detail in the APHA reference method for Enterococci:

a) Membrane Filtration (MF): The MF test has limitations, especially for turbid waters. Non-turbid water samples are passed through a 0.45 µm membrane filter which is placed on a selective medium (mE agar) for incubation at 41 ± 0.5°C for 48 ± 4 hours for growth of enterococci.

False positive samples are frequent by the MF method. Positive colonies must be confirmed as enterococci by positive esculin hydrolysis on esculin iron agar.

b) Enzyme Substrate: This method uses the fluorogenic substrate 4-methylumbelliferyl β-D-glucosidase (4-MUG). The β-D-glucosidase enzyme hydrolyzes the substrate, yielding a fluorescent signal when excited by long wavelength (365 to 366 nm) UV light. Non-enterococcus bacteria that produce β-D-glucosidase, such as some species of the genera Serratia, Kelbsiella, and Aerococcus are suppressed and will not produce positive results as long as their concentrations do not exceed 105 CFU/100 mL.

Seawaters require a 10x dilution prior to analysis to avoid interference from certain Bacillus spp.

Samples are incubated at 41 ± 0.5°C for 24 hours. Confirmation of positive test results is not needed by the enzyme substrate method.

MDL(s) and EMS Method & Analyte Code(s)*

Method Version / Analyte	Approx. MDL	EMS Analyte / Method Codes*
MF - Quantitative		
Enterococci	1 CFU /100 mL	0148 / X022
Enzyme Substrate Multi-well / Quanti-tray		
Enterococci	1 MPN /100 mL	0148 / not available

*Refer to EMS Parameter Dictionary on the Ministry of Environment and Climate Change Strategy website for all current EMS codes.

Matrix

Water

Interferences and Precautions

Refer to the method summaries above and to the applicable reference methods.

Follow all instructions in the prescriptive reference methods, including safety precautions and including confirming positive colonies to prevent reporting false positive test results for the MF test.

Refer to the manufacturer's instructions for the Enzyme Substrate test.

Work aseptically to prevent contamination of lab personnel and the lab area, and to prevent cross-contamination between samples. Refer to the *Government of Canada Canadian Biosafety Standard* for more information.

Sample Handling and Preservation

The sample is collected in the field and submitted unfiltered in a sterilized bacteriology bottle containing sufficient sodium thiosulfate to neutralize up to 15 mg/L residual chlorine, or a minimum of 10 mg anhydrous / 120 mL container or 15 mg of the pentahydrate form. Sodium Thiosulfate is effective in neutralizing the bactericidal effect of chlorine, neutralizing residual halogens, and preventing continuation of bactericidal action during sample transit.

Holding Time: Begin incubation within 24 hours of sample collection. Minimum volume required for analysis is 100 mL (APHA 9060A 2013).

Storage: Samples should be kept cool (at <10°C) during transport and storage until analysis. Do not freeze samples (APHA 9060B, 2013).

Procedure

Refer to detailed instructions provided within the following reference methods and supporting information for the Membrane Filtration and Fluorogenic Substrate Tests for guidance on the execution of these tests:

a) APHA 9230 Fecal Enterococcus/Streptococcus Groups.
b) APHA 9230 Section A Introduction.
c) APHA 9230 Section C Membrane Filtration Techniques.
d) APHA 9230 Section D Fluorogenic Substrate Test.
e) Enzyme Substrate Multi-well / Quanti-tray test: Refer to manufacturer's instructions.

The APHA guidance and the manufacturer's guidance for the enzyme substrate method are prescriptive and must be followed without modification.

Where subsampling occurs, be sure to homogenize the sample well prior to sub-sampling.

Incubation temperatures and times are important to prevent false positive and false negative reactions. Incubation instructions are provided by the media manufacturer and must be followed.

For data analysis, refer to reading instructions in the applicable reference documents as listed above.

Quality Control

Summary of QC Requirements		
QC Component	**Minimum Frequency**	**Minimum Data Quality Objectives**
Method Blank (MB)	One per batch (max 20 samples)	Less than reported DL
Lab Duplicates (DUP)	1 per batch (max 20 samples)[1]	± 65% RPD
Positive & Negative Controls	One each per day per incubator	Expected reaction to confirm proper operation of incubator and performance of the test.

[1] B.C. EWQA Program QC requirements for drinking water testing are more stringent, requiring duplicate samples at a frequency of 1 in 10 samples.

If DQOs are not met, repeat testing or report qualified test results.

Method Blank: The method blank is 100 mL sterile water that proceeds through the same sample handling processes as test samples, (including sodium thiosulfate if used with test samples; recommend preparing Method Blank in a sample bottle).

Laboratory Duplicates: Sample duplicates are prepared when sufficient sample is received to subsample for laboratory duplicates. Homogenize the sample well prior to subsampling. Process both aliquots through the same sample handling processes as test samples.

Positive / Negative Controls: Two are recommended for MF, three for the Enzyme Substrate test. Using the recommended organisms each day confirms that the test is performing as expected for all target and non-target organisms and that the incubator is operating as expected (gets to the right temperature at the right rate). Refer to APHA 9020 and Enzyme Substrate manufacturer's instructions for more information.

Proofing of sample bottles, organisms, reagents, and supplies by lot is recommended to demonstrate sterility and performance prior to use. Refer to APHA 9020 for more information on recommended Quality Control practices for this test.

References

1. APHA 9230 (2013) Fecal Enterococcus / Streptococcus Groups.
2. APHA 9060 (2013) Samples.
3. APHA 9020 (2015) Quality Control.
4. Enzyme Substrate Multi-well / Quanti-tray Manufacturer's Instructions.

Revision History

Feb 14, 1994	Publication in 1994 Lab Manual
Nov 14, 2002	SEAM codes replaced by EMS codes
Dec 20, 2019	Updated to BC Lab Manual Prescriptive Method format. Prescriptive nature of test is confirmed. Enzyme Substrate test procedure option was added. APHA 9230 was revised in 2013. APHA 9060 was revised in 2013. APHA 9020 was revised in 2015. QC Section updated to include Method Blanks and Duplicate Samples. Changed sample storage temperature to <10°C as per APHA 9060 (2013).

Salmonella in Solids by Multiple Tube Fermentation (MTF) – Prescriptive

Parameter	Salmonella
Analytical Method	Multiple Tube Fermentation (MTF)
Introduction	This method is prescriptive. The Multiple Tube Fermentation method is used to derive a probability estimate (i.e. Most Probable Number, MPN) of the number of salmonella in soil, sludge or other solids. Options are provided for initial solid sample preparation and homogenization. Salmonella belongs to the Family Enterobacteriaceae and are Gram negative, motile, non-spore forming rods that ferment glucose but not lactose. Salmonella are enteric pathogens which can cause gastroenteritis. Salmonella are the most common cause of bacterial diarrhea in North America, and are carried in the intestinal tracts of many animal species and humans. Composted sewage sludge must be proven to be salmonella free before being transported or used as a soil additive. A licence must be obtained from the Public Health Agency of Canada (PHAC) to purchase the control organisms required for this test. Refer to the PHAC website.
Method Summary	Salmonella are grown in enrichment broth (Tetrathionate broth) and are confirmed by isolation on selective media (XLD agar) and subsequent serotyping with specific Salmonella "O" antisera and biochemical reactions in API 20E® strips (BioMerieux).

MDL(s) and EMS Method & Analyte Code(s)*

Analyte	Approx. MDL	EMS Analyte / Method Codes*
Salmonella	1 MPN / gram	0451 /

*Refer to EMS Parameter Dictionary on the Ministry of Environment and Climate Change Strategy website for all current EMS codes.

Matrix	Solids (Soil, Sludge)
Interferences and Precautions	There are limitations in the sensitivity and selectivity of isolation procedures for the detection of the more than 1700 salmonella serotypes currently recognized. A negative result may not imply the absence of salmonella. Media formulations will vary to a small degree between manufacturers and between lots. These variations are minor and will not impact the reliability of the test method. Work aseptically to prevent contamination of lab personnel and the lab area, and to prevent cross-contamination between samples. Refer to the Government of Canada Canadian Biosafety Standard for more information. Incubation temperatures and times are important to prevent false positive and false negative reactions. Incubation instructions are provided by the media manufacturer and must be followed. Where subsampling occurs, be sure to homogenize the sample well prior to sub-sampling.

Sample Handling and Preservation

Samples are collected in the field and submitted unpreserved in a sterilized bottle or Whirl-Pak™ bag (or equivalent). Minimum weight required for analysis is 75 grams of solids.

Holding Time: Begin incubation no later than 48 hours after sample collection (historical instructions; no reference).

Storage: Samples should be kept cool (at <10°C) during transport and storage until analysis. Do not freeze samples (APHA 9060B, 2013).

Apparatus and Materials

a) Incubator capable of maintaining 35°C ± 0.5°C.
b) Sterile disposable serological pipettes, 1.0 mL and 10 mL.
c) Tetrathionate broth base.
d) XLD agar.
e) BHI agar.
f) 18 x 150 mm test tubes with stainless steel closures.
g) 20 x 150 mm test tubes with stainless steel closures.
h) Autoclave for steam sterilization of glassware and media.
i) Bunsen burner.
j) Platinum or disposable plastic inoculation loops, 3 mm diameter.
k) Gram staining reagents (available commercially from Difco).
l) Microscope slides and microscope with oil immersion lens.
m) Buffered water dilution blanks, 10 mL in 20 x 150 mm test tubes.
n) Petri dishes, sterile, disposable, 100 x 15 mm.
o) API 20E® strips (BioMerieux).
p) Salmonella O Grouping Antisera (BBL).
q) Laminar flow biohazard hood.
r) Solid sample preparation materials:
   1st option: Sterile Stomacher® bags.
   2nd option (CSSS reference): dilution bottles and optional mechanical shaker.

Reagents

The exact formulation of media types (d) through (i) may vary from time to time and between manufacturers. The formulations and instructions below are provided as a guideline. Purchase media with the correct components and with similar ratios, and follow the manufacturer's instructions for preparation, and for quality control testing of the media.

a) STOCK PHOSPHATE (PO4) BUFFER SOLUTION:

Dissolve 34 g potassium dihydrogen phosphate (KH2PO4) in 500 mL deionized water (DI). Adjust to pH 7.2 ± 0.1 with 1N sodium hydroxide (NaOH), and dilute to 1 L with DI. Filter through a sterile 0.22 µm pore size membrane filter into a sterile amber bottle or autoclave for 15 minutes at 121°C. Store at 2-8°C. Discard if solution becomes turbid.

b) STOCK MAGNESIUM CHLORIDE SOLUTION:

Dissolve 38 g magnesium chloride (MgCl2) in 1L DI. Filter through a sterile 0.22 µm pore size membrane filter into a sterile amber bottle or autoclave for 15 minutes at 121°C. Store at 2-8°C. Discard if solution becomes turbid. No recommended final pH. Store at 2-8°C. Discard if solution becomes turbid.

c) BUFFERED DILUTION WATER:

Add 1.25 mL stock PO4 buffer solution and 5mL stock MgCl2 solution to a 1 L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

Dilution blanks:	10 mL in 20 x 150mm test tubes
	90 mL in milk dilution bottles

Autoclave 10 – 90 mL volumes at 121°C for 15 minutes. Test for sterility. Final volume should be 10 mL ± 2% and 90 mL ± 2%. Final pH should be 7.2 + 0.1 at 25°C. Store at room temperature for up to 1 month.

d) TETRATHIONATE BROTH BASE (DIFCO) SINGLE STRENGTH:

Formula (grams per litre):

i)	Proteose peptone	5.0 g
	Bacto bile salts	1.0 g
	Sodium thiosulfate	30.0 g
	Calcium carbonate	10.0 g
ii)	Iodine-iodide solution	
	Iodine crystals*	6.0 g
	Potassium Iodide	5.0 g
	Deionized water	20.0 mL

Grind iodine crystals with potassium iodide with a mortar and pestle. Dissolve in DI.

***Note**: Crystalline iodine is extremely volatile and corrosive. Do not use foil to cover beaker. Use fume hood and wear gloves when handling crystalline iodine.*

Suspend 46 g powdered medium in 1 L deionized water and heat to boiling. Cool below 60°C. Add 20 mL iodine-iodide solution per litre of tetrathionate broth base. Do not heat medium after adding iodine. Dispense 10 mL quantities into sterile 18 x 150 mm screw cap test tubes. Use medium the same day it is prepared. Final pH should be 8.4 ± 0.2 at 25°C or as per manufacturer's instructions. Test media as per manufacturer's instructions. Incubate at 35°C $\pm$ 2°C for 18-24 hours. Do not store after iodine-iodide solution has been added. Use media immediately. Media without iodine-iodide solution can be stored at 2-8°C for 3 months in screw cap tubes.

e) TETRATHIONATE BROTH BASE - DOUBLE STRENGTH:

Formula is as listed above.

Suspend 46 g in 500 mL deionized water and proceed as instructed above, using 40 mL iodine-iodide solution per litre. Dispense 20 mL quantities into sterile 25 x 150 mm screw cap test tubes. Final pH should be 8.4 ± 0.2 at 25°C or as per manufacturer's instructions. Test media as per manufacturer's instructions. Incubate at 35°C $\pm$ 2°C for 18-24 hours. Do not store after iodine-iodide solution has been added. Use media immediately. Media without iodine-iodide solution can be stored at 2-8°C for 3 months in screw cap tubes.

f) XLD AGAR, dehydrated (DIFCO):

Formula (grams per litre):

Bacto yeast extract	3.00 g
L-lysine	5.00 g
Xylose	3.75 g
Lactose	7.50 g
Saccharose	7.50 g
Sodium desoxycholate	2.50 g
Ferric ammonium citrate	0.80 g
Sodium thiosulfate	6.80 g
Sodium chloride	5.00 g
Bacto agar	15.0 g
Phenol red	0.08 g

Suspend 55g in 1 L deionized water and boil to dissolve completely. Avoid

overheating. Do not autoclave. Cool to 45 to 50°C and dispense 17-20 mL into sterile 100 x 15mm petri plates. Final pH should be 7.4 ± 0.2 at 25°C or as per manufacturer's instructions. Test media as per manufacturer's instructions. Incubate at 35°C ± 2°C for 24 hours. Store at 2-8°C for 2 weeks. Protect from light.

g) BRAIN HEART INFUSION AGAR, dehydrated (BHIA), DIFCO:

Formula (grams per litre):

Calf brain infusion	200 g
Beef heart infusion	250 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Bacto agar	15.0 g

Suspend 52 g of powdered medium in 1 L deionized water and heat to boiling to dissolve completely. Sterilize in the autoclave for 15 minutes at 121°C. Dispense 17-20 mL into sterile 100 x 15 mm petri plates. Final pH should be 7.4 ± 0.2 at 25°C or as per manufacturer's instructions. Test media as per manufacturer's instructions. Incubate at 35°C ± 0.5°C for 24 hours.

h) NORMAL SALINE:

Sodium chloride	8.5 g
Deionized water	1.0 L

Dissolve NaCl in DI and autoclave 90mL aliquots in milk dilution bottles for 15 minutes at 121°C. Test for sterility. Final volume should 90 mL ± 2%. Final pH should be 7.2 + 0.1 at 25°C. Store at room temperature for up to 1 month.

i) 0.1% PEPTONE IN BUFFERED WATER:

Proteose peptone	1.0 g
Stock buffered water	1.0 L

Dissolve peptone in buffered water and heat to dissolve. Dispense in 90 mL aliquots in milk dilution bottles or in 10 mL aliquots in test tubes. Autoclave for 15 minutes at 121°C. Test for sterility. Final pH should be 7.2 + 0.2 at 25°C.

Procedure

Detailed, instructions for the MTF technique are provided in APHA 9221 B.

a) Set up test tube rack with a sequence of test tubes which includes at least 5 replicate tubes per sample volume and 3 dilutions.
b) Use double strength tetrathionate broth tubes for the initial sample volume of 10 mL per tube. Use single strength tetrathionate broth tubes for all subsequent sample volumes.
c) Prepare soil samples using one of the two following procedures:
   Option 1: Weigh 10 g of as-received soil or sludge (not dried) into a sterile Stomacher® bag. Make sure there are no rocks or hard pieces of wood included. Add 90 mL 0.1% peptone in buffered water. Mix by "paddling" for 30 seconds in Stomacher® bag.
   Option 2 (CSSS reference): Pass as-received soil sample (not dried) through a 2 mm mesh sieve and mix thoroughly. Weigh a 10 g soil portion into a dilution bottle containing 95 mL diluent. Sterile glass beads (~ 25 x 2 mm) may be added to aid in mixing. Cap the bottle, shake on a mechanical shaker for 10 minutes or shake by hand in 45° arc at least 50 times. Re-shake each sample vigorously prior to subsampling
d) Keep solids in suspension and dispense 10 mL aliquots into each of 5 double strength tetrathionate broth tubes, 1 mL aliquots into each of 5 single strength

tetrathionate broth tubes, and decimal dilutions as required by serially diluting 1 mL aliquots in 10 mL buffered water blanks and inoculating single strength tetrathionate broth tubes.

e) Incubate tubes for 48 ± 3 hours at 35± 0.5°C.
f) Transfer a loopful of each culture to sections marked off on plates of XLD agar. Streak for isolated colonies. Incubate inverted agar plates at 35 ± 0.5°C for 24 ± 2 hours.
g) Pick any red colonies with black centers to purify on BHIA and incubate for 18-24 hours at 35 ± 0.5°C.
h) Mark off two ovals on a microscope slide and place a drop of saline and a drop of polyvalent "O" antiserum in either oval. Make a suspension of a well-isolated colony from BHIA first in the saline and then in the antiserum. Continue rocking the slide for 1-2 minutes to insure adequate mixing. Salmonella spp. will produce a strong positive agglutination in the polyvalent "O" antiserum with no agglutination in the saline control. If there is a questionable agglutination, specific antisera may be screened. Each Salmonella sp. will react with only one specific "O" antiserum in addition to the polyvalent antiserum. If the saline control agglutinates the organism is self-agglutinating or rough, and must be confirmed by biochemical reaction.
i) For biochemical confirmation of Salmonella spp. make a suspension in saline and proceed according to the instructions for inoculating and incubating an API 20E® strip.
j) Count only those tubes containing growth subsequently found to be Salmonella for calculations. Note that Proteus sp., Citrobacter sp. and Arizona sp. can sometimes resemble Salmonella sp. on XLD agar. Only Salmonella will agglutinate with polyvalent "O" antiserum. The biochemical profile of each genus is specific.

Note 1: All Salmonella are potentially pathogenic. All plates and suspensions must be handled in a biohazard hood. All refuse and spent media must be autoclaved for 30 minutes at 121°C before disposal.

Note 2: Use and handling instructions for control organisms, and quality control practice guidelines are not described in the manufacturer's instructions. Refer to APHA 9020 for guidance on these topics.

DATA ANALYSIS

Refer to a standard MPN index (e.g. Section 4.0 of the Laboratory Quality Assurance/Quality Control section of the Microbiology Examination Section of the laboratory manual) for guidance regarding calculation of bacterial density. Note that a minimum of 3 dilutions are required for this calculation.

Also refer to:

i) APHA 9221 for details of MTF interpretation and MPN conversion.
ii) APHA 9260B for information on Salmonella quantitation tests.

Test results should be reported in units of CFU per gram of wet (as-received) soil.

Quality Control

Summary of QC Requirements		
QC Component	**Minimum Frequency**	**Minimum Data Quality Objectives**
Method Blank (MB)	One per batch (max 20 samples)	Less than reported DL
Lab Duplicates (DUP)	1 per batch (max 20 samples)	± 65% RPD
Positive & Negative Controls	One each per day per incubator	Expected reaction to confirm proper operation of incubator and performance of the test.
If DQOs are not met, repeat testing or report qualified test results.		

Method Blank: The Method Blank consists of the media / reagents used in sample preparation that proceeds through the same sample handling processes as test samples. It is recommended for the Method Blank to be prepared using a sample bottle/container.

Laboratory Duplicates: Sample duplicates are prepared when sufficient sample is received to subsample for laboratory duplicates. Homogenize the sample well prior to subsampling. Process both aliquots through the same sample handling processes as test samples.

Positive / Negative Controls: Two are recommended. Using both each day confirms that the test is performing as expected for all target and non-target organisms and that the incubator is operating as expected (reaches correct temperature at correct rate). Refer to APHA 9020 for more information.

Proofing of sample bottles, organisms, reagents, and supplies by lot is recommended to demonstrate sterility and performance prior to use. Refer to APHA 9020 for more information on recommended Quality Control practices for this test.

References

1. APHA 9221 (2014) Multiple-Tube Fermentation Technique for Members of the Coliform Group.
2. APHA 9260 (2007) Detection of Pathogenic Bacteria.
3. Canadian Society of Soil Science (CSSS), Soil Sampling and Methods of Analysis, 2nd Edition, edited by M.R. Carter, E.G. Gregorich.
4. APHA 9060 (2013) Samples.
5. APHA 9020 (2015) Quality Control.

Revision History

Feb 14, 1994	Publication in 1994 Lab Manual
Nov 14, 2002	SEAM codes replaced by EMS codes
Dec 20, 2019	Updated to BC Lab Manual Prescriptive Method format. Prescriptive nature of test is confirmed. APHA 9221 was revised in 2014. APHA 9260 was revised in 2007. APHA 9060 was revised in 2013. APHA 9020 was revised in 2015. Added simpler, alternative soil preparation option with CSSS reference. QC Section updated to include Method Blanks and Duplicate Samples. Changed sample storage temperature to <10°C as per APHA 9060 (2013).

Salmonella in Fresh Water, Wastewater, and Marine Water by Multiple - Tube Technique (MPN)

Parameter

Salmonella

Analytical Method and EMS Codes

Confirmed MPN: **SALM X015**

Scope

This method describes the probability estimation of the numbers of salmonella from fresh water, wastewater, and marine water. Salmonella are grown in enrichment broth (Tetrathionate broth) and confirmed by isolation on selective media (XLD agar) and subsequent serotyping with specific Salmonella "O" antisera and biochemical reactions in API 20E® strips (Analytab Products).

Principle

Salmonella belong to the Family Enterobacteriaceae and are Gram negative, motile, non-sporeforming rods that ferment glucose but not lactose. Salmonella are enteric pathogens which can cause gastroenteritis. Salmonella are the most common cause of bacterial diarrhoea in North America, and are carried in the intestinal tracts of many animal species and humans. Although salmonella are long-lived in the environment, they are often difficult to document due to their low numbers in receiving waters.

Sample Handling

The sample is collected in the field and submitted unfiltered and unpreserved in a sterilized water bacteriology bottle. Chlorinated water samples should be treated with sodium thiosulfate (final concentration 0.01% w/v) to neutralize the bactericidal effect of chlorine. The sample should be kept at 4°C until analysis. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum volume required for analysis is 75 mL.

Detection Limit

2 MPN/100mL

Interferences

The occurrence of salmonella in water is highly variable and there are limitations in the sensitivity and selectivity of isolation procedures for the detection of the more than 1700 salmonella serotypes currently recognized. A negative result may not imply the absence of salmonella. Residual chlorine in chlorinated water must be neutralized with sodium thiosulfate at time of sampling.

Precision

There are no standard reference materials for salmonella. Mean recovery of American Type Culture Collection (ATCC) 14028 S. typhimurium in tetrathionate broth confirmed on XLD agar is 80% and 125% for seeded inocula of 1730 and 20.3 CFU/100mL with coefficients of variation of 23% and 18% respectively.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 35°C ± 0.5°C temperature.
b) Sterile disposable serological pipettes, 1 mL and 10mL.
c) Tetrathionate broth base.
d) XLD agar.
e) BHI agar.

f) 18mm test tubes with stainless steel closures.
g) 20mm test tubes with stainless steel closures.
h) Autoclave for steam sterilization of glassware and media.
i) Bunsen burner.
j) Platinum inoculation loops, 3mm diameter.
k) Gram staining reagents (available commercially from Difco).
l) Microscope slides and microscope with oil immersion lens.
m) Buffered water dilution blanks, 10mL in 20mm test tubes.
n) Petri dishes, sterile disposable, 100 x 15mm.
o) API 20E® strips (API Analytab Products).
p) Salmonella O Grouping Antisera (BBL).
q) Laminar flow biohazard hood.

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION.

Dissolve 34.0g of potassium dihydrogen phosphate (KH_2PO_4) in 500mL deionized water (DI). Adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM CHLORIDE SOLUTION.

Dissolve 38g magnesium chloride ($MgCl_2$) in 1L DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

c) BUFFERED DILUTION WATER.

Add 1.25mL stock PO_4 buffer solution and 5mL stock $MgCl_2$ solution to a 1L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

Dilution blanks: 10mL in 20mm test tubes
100mL in milk dilution bottles
Autoclave 10 - 100mL volumes at 121°C for 15 minutes.

d) TETRATHIONATE BROTH BASE (DIFCO) SINGLE STRENGTH

Formula (grams per litre):

i) Proteose peptone	5.0 g
Bacto bile salts	1.0 g
Sodium thiosulfate	30.0 g
Calcium carbonate	10.0 g
ii) Iodine-iodide solution	
Iodine crystals*	6.0 g
Potassium Iodide	5.0 g
DI	20.0 mL

Grind iodine crystals with potassium iodide with a mortar and pestle. Dissolve in DI.

***Note**: Crystalline iodine is extremely volatile and corrosive. Do not use foil to cover beaker. Use fume hood and wear gloves when handling crystalline iodine.*

Suspend 46g powdered medium in 1L deionized water and heat to boiling. Cool below 60°C. Add 20mL iodine-iodide solution per litre tetrathionate broth base. Do not heat medium after adding iodine. Dispense 10mL quantities into sterile test tubes. Use medium the same day it is prepared. Final pH should be 8.4 ± 0.2 at 25°C.

d) TETRATHIONATE BROTH BASE - DOUBLE STRENGTH:

Formula as listed above.
Suspend 46g in 500mL deionized water and proceed as instructed above, using 40mL iodine-iodide solution per litre.

f) XLD AGAR, dehydrated (DIFCO)

Formula (grams per litre):	
Bacto yeast extract	3.00 g
L-lysine	5.00 g
Xylose	3.75 g
Lactose	7.50 g
Saccharose	7.50 g
Sodium desoxycholate	2.50 g
Ferric ammonium citrate	0.80 g
Sodium thiosulfate	6.80 g
Sodium chloride	5.00 g
Bacto agar	15.00 g
Phenol red	0.08 g

Suspend 57g in 1L deionized water and heat to dissolve completely. Avoid overheating. Do not autoclave. Cool to 55°C and dispense 17 - 20mL into sterile petri plates. Final pH 7.4 ± 0.2 at 25°C.

g) BRAIN HEART INFUSION AGAR, dehydrated (BHIA) (DIFCO)

Formula (grams per litre):	
Calf brain infusion	200.0 g
Beef heart infusion	250.0 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Bacto agar	15.0 g

Suspend 52g powdered medium in 1L deionized water and heat to boiling to dissolve completely. Autoclave for 15 minutes at 121°C.

h) NORMAL SALINE

Sodium Chloride	8.5 g
DI	1.0 L

Dissolve NaCl in DI and autoclave 100mL aliquots in milk dilution bottles for 15 minutes at 121°C.

Procedure

a) Set up test tube rack with a sequence of test tubes which includes at least 5 replicate tubes per sample volume and 3 dilutions. Wastewater samples will require additional dilutions.

b) Use double strength tetrathionate broth tubes for the initial sample volume of 10mL per tube. Use single strength tetrathionate broth tubes for all subsequent sample volumes.

c) Shake the sample bottle vigorously about 30 times and measure the desired volume of the sample into the tubes of tetrathionate broth. Use 10mL buffered water blanks to make decimal dilutions of sample for inoculation.

d) If required, filter 50-100mL non-turbid sample through a 0.45µm filter and add filter directly to a tube of single strength tetrathionate broth.

e) Incubate tubes for 48 hours at 35°C.

f) Transfer a loopful of each culture to sections marked off on plates of XLD agar. Streak for isolated colonies. Incubate inverted agar plates at 35°C for 24 hours.

g) Pick any red colonies with black centres to purify on BHIA and incubate for 18-24 hours at 35°C.

h) Mark off two ovals on a microscope slide and place a drop of saline and a drop of polyvalent "O" antiserum in either oval. Make a suspension of a well-isolated colony from BHIA first in the saline and then in the antiserum. Continue rocking the slide to insure adequate mixing for 1-2 minutes. Salmonella spp. will produce a strong positive agglutination in the polyvalent "O" antiserum with no agglutination in the saline control. If there is a questionable agglutination, specific antisera may be screened. Each Salmonella sp. will react with only one specific "O" antiserum in addition to the polyvalent antiserum. If the saline control agglutinates, the organism is self-agglutinating or rough, and must be confirmed by biochemical reaction.

i) For biochemical confirmation of Salmonella spp. make a suspension in saline and proceed according to the instructions for inoculating and incubating an API 20E strip.

j) Count only those tubes containing growth subsequently found to be salmonella for calculations. Note that Proteus sp., Citrobacter sp. and Arizona sp. can sometimes resemble Salmonella sp. on XLD agar. Only salmonella will agglutinate with polyvalent "O" antiserum. The biochemical profile of each genus is specific. Refer to a standard MPN index (Section 4.6 of the Microbiological Quality Assurance/Quality Control section of this manual) for calculation of bacterial density. Note that a minimum of 3 dilutions are required for this calculation. Use the set of dilutions which includes the highest dilution with all positive tubes and the next two higher dilutions.

*Note that all Salmonella are potentially pathogenic. All plates and suspensions must be handled in a biohazard hood. All refuse and spent media must be autoclaved for 45 minutes at 121°C before disposal.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9260.

b) Bartlett, K.H. and Trust, T.J. "Isolation of Salmonella and Other Potential Pathogens from the Freshwater Aquarium Snail Amullaria." Applied and Environmental Microbiology 31: 635-639, 1976.

c) Edgar, D. and Soar, M.S. "Evaluation of Culture Media for the Isolation of Salmonellas from Sewage Sludge." Journal of Applied Bacteriology 47: 237-241, 1979.

Revision History

February 14, 1994:	Publication in 1994 Laboratory Manual.
November 14, 2002:	SEAM Codes replaced by EMS codes.

Salmonella in Solids by Multiple Tube Technique (MPN)

Parameter

Salmonella

Analytical Method and EMS codes

Confirmed MPN : **SALM X390**

Scope

This method describes the probability estimation of the numbers of salmonella from soils, sludge or other solids. Salmonella are grown in enrichment broth (Tetrathionate broth) and confirmed by isolation on selective media (XLD agar) and subsequent serotyping with specific Salmonella "O" antisera and biochemical reactions in API 20E® strips (Analytab Products).

Principle

Salmonella belong to the Family Enterobacteriaceae and are Gram negative, motile, non-sporeforming rods that ferment glucose but not lactose. Salmonella are enteric pathogens which can cause gastroenteritis. Salmonella are the most common cause of bacterial diarrhoea in North America, and are carried in the intestinal tracts of many animal species and humans. Composted sewage sludge must be proven to be salmonella free before being transported or used as a soil additive.

Sample Handling

The sample is collected in the field and submitted unpreserved in a sterilized bottle or Whirl-Pak™ bag. The sample should be kept at 4°C until analysis. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum weight required for analysis is 75 grams.

Detection Limit

2 MPN/gram

Interferences

There are limitations in the sensitivity and selectivity of isolation procedures for the detection of the more than 1700 salmonella serotypes currently recognized. A negative result may not imply the absence of salmonella.

Precision

There are no standard reference materials for salmonella. Mean recovery of American Type Culture Collection (ATCC) 14028 S.typhimurium in tetrathionate broth confirmed on XLD agar is 80% and 125% for seeded inocula of 1730 and 20.3 CFU/100mL with coefficients of variation of 23% and 18%, respectively.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 35°C ± 0.5°C temperature.
b) Sterile disposable serological pipettes, 1mL and 10mL.
c) Tetrathionate broth base.
d) XLD agar.
e) BHI agar.
f) 18mm test tubes with stainless steel closures.
g) 20mm test tubes with stainless steel closures.
h) Autoclave for steam sterilization of glassware and media.
i) Bunsen burner.
j) Platinum inoculation loops, 3mm diameter.
k) Gram staining reagents (available commercially from Difco).
l) Microscope slides and microscope with oil immersion lens.

m) Buffered water dilution blanks, 10mL in 20mm test tubes.
n) Petri dishes, sterile, disposable, 100 x 15mm.
o) API 20E® strips (API Analytab Products).
p) Salmonella O Grouping Antisera (BBL).
q) Laminar flow biohazard hood.
r) Stomacher®.
s) Sterile Stomacher® bags (Canlab).

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION.

Dissolve 34.0g potassium dihydrogen phosphate (KH_2PO_4) in 500mL deionized water (DI). Adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM CHLORIDE SOLUTION.

Dissolve 38g magnesium chloride ($MgCl_2$) in 1L DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

c) BUFFERED DILUTION WATER.

Add 1.25mL stock PO_4 buffer solution and 5mL stock $MgCl_2$ solution to a 1L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

Dilutions blanks: 10mL in 20mm test tubes
100mL in milk dilution bottles
Autoclave 10 - 100mL volumes at 121°C for 15 minutes.

d) TETRATHIONATE BROTH BASE (DIFCO) SINGLE STRENGTH

Formula (grams per litre):

i)	Proteose peptone	5.0 g
	Bacto bile salts	1.0 g
	Sodium thiosulfate	30.0 g
	Calcium carbonate	10.0 g
ii)	Iodine-iodide solution	
	Iodine crystals*	6.0 g
	Potassium Iodide	5.0 g
	DI	20.0 mL

Grind iodine crystals with potassium iodide with a mortar and pestle. Dissolve in DI.

***Note**: Crystalline iodine is extremely volatile and corrosive. Do not use foil to cover beaker. Use fume hood and wear gloves when handling crystalline iodine.*

Suspend 46g powdered medium in 1L deionized water and heat to boiling. Cool below 60°C. Add 20mL iodine-iodide solution per litre of tetrathionate broth base. Do not heat medium after adding iodine. Dispense 10mL quantities into sterile test tubes. Use medium the same day it is prepared. Final pH should be 8.4 ± 0.2 at 25°C.

e) TETRATHIONATE BROTH BASE - DOUBLE STRENGTH:

Formula as listed above.

Suspend 46g in 500mL deionized water and proceed as instructed above, using 40mL iodine-iodide solution per litre.

f) XLD AGAR, dehydrated (DIFCO)

Formula (grams per litre):	
Bacto yeast extract	3.00 g
L-lysine	5.00 g
Xylose	3.75 g
Lactose	7.50 g
Saccharose	7.50 g
Sodium desoxycholate	2.50 g
Ferric ammonium citrate	0.80 g
Sodium thiosulfate	6.80 g
Sodium chloride	5.00 g
Bacto agar	15.00 g
Phenol red	0.08 g

Suspend 57g in 1L deionized water and boil to dissolve completely. Avoid overheating. Do not autoclave. Cool to 55°C and dispense 17-20mL into sterile petri plates. Final pH should be 7.4 ± 0.2 at 25°C.

g) BRAIN HEART INFUSION AGAR, dehydrated (BHIA) (DIFCO)

Formula (grams per litre):	
Calf brain infusion	200.0 g
Beef heart infusion	250.0 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Bacto agar	15.0 g

Suspend 52g of powdered medium in 1L deionized water and heat to boiling to dissolve completely. Sterilize in the autoclave for 15 minutes at 121°C.

h) NORMAL SALINE

Sodium chloride	8.5 g
DI	1.0 L

Dissolve NaCl in DI and autoclave 100mL aliquots in milk dilution bottles for 15 minutes at 121°C.

i) 0.1% PEPTONE IN BUFFERED WATER

Proteose peptone	1.0 g
Stock buffered water	1.0 L

Dissolve peptone in buffered water and heat to dissolve. Dispense in milk dilution bottles or in 10mL amounts in test tubes. Autoclave for 15 minutes at 121°C.

Procedure

a) Set up test tube rack with a sequence of test tubes which includes at least 5 replicate tubes per sample volume and 3 dilutions.

b) Use double strength tetrathionate broth tubes for the initial sample volume of 10mL per tube. Use single strength tetrathionate broth tubes for all subsequent sample volumes.

c) Weigh 10g soil or sludge into a sterile Stomacher® bag. Make sure there are no rocks or hard pieces of wood included. Add 100mL 0.1% peptone in buffered water. Mix by "paddling" for 30 seconds in Stomacher®. Keep solids in suspension and dispense 10mL aliquots into each of 5 double strength tetrathionate broth tubes, 1mL aliquots into each of 5 single strength tetrathionate broth tubes, and decimal dilutions as required by serially diluting 1mL aliquots in 10mL buffered water blanks and inoculating single strength tetrathionate broth tubes.

d) Incubate tubes for 48 hours at 35°C.

e) Transfer a loopful of each culture to sections marked off on plates of XLD agar. Streak for isolated colonies. Incubate inverted agar plates at 35°C for 24 hours.

f) Pick any red colonies with black centres to purify on BHIA and incubate for 18-24 hours at 35°C.

g) Mark off two ovals on a microscope slide and place a drop of saline and a drop of polyvalent "O" antiserum in either oval. Make a suspension of a well-isolated colony from BHIA first in the saline and then in the antiserum. Continue rocking the slide for 1-2 minutes to insure adequate mixing. Salmonella spp. will produce a strong positive agglutination in the polyvalent "O" antiserum with no agglutination in the saline control. If there is a questionable agglutination, specific antisera may be screened. Each Salmonella sp. will react with only one specific "O" antiserum in addition to the polyvalent antiserum. If the saline control agglutinates the organism is self-agglutinating or rough, and must be confirmed by biochemical reaction.

h) For biochemical confirmation of Salmonella spp. make a suspension in saline and proceed according to the instructions for inoculating and incubating an API 20E® strip.

i) Count only those tubes containing growth subsequently found to be salmonella for calculations. Note that Proteus sp., Citrobacter sp. and Arizona sp. can sometimes resemble Salmonella sp. on XLD agar. Only salmonella will agglutinate with polyvalent "O" antiserum. The biochemical profile of each genus is specific. Refer to a standard MPN index (section 4.0 of the Laboratory Quality Assurance/Quality Control section of this manual) for calculation of bacterial density. Note that a minimum of 3 dilutions are required for this calculation. Use the set of dilutions which includes the highest dilution with all positive tubes and the next two higher dilutions.

Note: All Salmonella are potentially pathogenic. All plates and suspensions must be handled in a biohazard hood. All refuse and spent media must be autoclaved for 45 minutes at 121°C before disposal.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9260.

b) Bartlett, K.H. and Trust, T.J. "Isolation of Salmonella and Other Potential Pathogens from the Freshwater Aquarium Snail Amullaria." Applied and Environmental Microbiology 31: 635-639, 1976.

c) Edgar, D. and Soar, M.S. "Evaluation of Culture Media for the Isolation of Salmonellas from Sewage Sludge." Journal of Applied Bacteriology 47: 237-241, 1979.

Revision History

February 14, 1994:	Publication in 1994 Laboratory Manual.
November 14, 2002:	SEAM codes replaced by EMS codes.

Salmonella in Solids by Multiple Tube Fermentation (MTF) – Prescriptive

Parameter

Salmonella

Analytical Method

Multiple Tube Fermentation (MTF)

Introduction

This method is prescriptive. The Multiple Tube Fermentation method is used to derive a probability estimate (i.e. Most Probable Number, MPN) of the number of salmonella in soil, sludge or other solids. Options are provided for initial solid sample preparation and homogenization.

Salmonella belong to the Family Enterobacteriaceae and are Gram negative, motile, non-spore forming rods that ferment glucose but not lactose. Salmonella are enteric pathogens which can cause gastroenteritis. Salmonella are the most common cause of bacterial diarrhea in North America, and are carried in the intestinal tracts of many animal species and humans. Composted sewage sludge must be proven to be salmonella free before being transported or used as a soil additive.

A licence must be obtained from the Public Health Agency of Canada (PHAC) to purchase the control organisms required for this test. Refer to the PHAC website.

Method Summary

Salmonella are grown in enrichment broth (Tetrathionate broth) and are confirmed by isolation on selective media (XLD agar) and subsequent serotyping with specific Salmonella "O" antisera and biochemical reactions in API 20E® strips (BioMerieux).

MDL(s) and EMS Method & Analyte Code(s)*

Analyte	Approx. MDL	EMS Analyte / Method Codes*
Salmonella	1 MPN / gram	0451 /

*Refer to EMS Parameter Dictionary on the Ministry of Environment and Climate Change Strategy website for all current EMS codes.

Matrix

Solids (Soil, Sludge)

Interferences and Precautions

There are limitations in the sensitivity and selectivity of isolation procedures for the detection of the more than 1700 salmonella serotypes currently recognized. A negative result may not imply the absence of salmonella.

Media formulations will vary to a small degree between manufacturers and between lots. These variations are minor and will not impact the reliability of the test method.

Work aseptically to prevent contamination of lab personnel and the lab area, and to prevent cross-contamination between samples. Refer to the Government of Canada Canadian Biosafety Standard for more information.

Incubation temperatures and times are important to prevent false positive and false negative reactions. Incubation instructions are provided by the media manufacturer and must be followed.

Where subsampling occurs, be sure to homogenize the sample well prior to sub-sampling.

Sample Handling and Preservation

Samples are collected in the field and submitted unpreserved in a sterilized bottle or Whirl-Pak™ bag (or equivalent). Minimum weight required for analysis is 75 grams of solids.

Holding Time: Begin incubation no later than 48 hours after sample collection (historical instructions; no reference).

Storage: Samples should be kept cool (at <10°C) during transport and storage until analysis. Do not freeze samples (APHA 9060B, 2013).

Apparatus and Materials

a) Incubator capable of maintaining 35°C ± 0.5°C.
b) Sterile disposable serological pipettes, 1.0 mL and 10 mL.
c) Tetrathionate broth base.
d) XLD agar.
e) BHI agar.
f) 18 x 150 mm test tubes with stainless steel closures.
g) 20 x 150 mm test tubes with stainless steel closures.
h) Autoclave for steam sterilization of glassware and media.
i) Bunsen burner.
j) Platinum or disposable plastic inoculation loops, 3 mm diameter.
k) Gram staining reagents (available commercially from Difco).
l) Microscope slides and microscope with oil immersion lens.
m) Buffered water dilution blanks, 10 mL in 20 x 150 mm test tubes.
n) Petri dishes, sterile, disposable, 100 x 15 mm.
o) API 20E® strips (BioMerieux).
p) Salmonella O Grouping Antisera (BBL).
q) Laminar flow biohazard hood.
r) Solid sample preparation materials:
   1st option: Sterile Stomacher® bags.
   2nd option (CSSS reference): dilution bottles and optional mechanical shaker.

Reagents

The exact formulation of media types (d) through (i) may vary from time to time and between manufacturers. The formulations and instructions below are provided as a guideline. Purchase media with the correct components and with similar ratios, and follow the manufacturer's instructions for preparation, and for quality control testing of the media.

a) STOCK PHOSPHATE (PO4) BUFFER SOLUTION:

   Dissolve 34 g potassium dihydrogen phosphate (KH2PO4) in 500 mL deionized water (DI). Adjust to pH 7.2 ± 0.1 with 1N sodium hydroxide (NaOH), and dilute to 1 L with DI. Filter through a sterile 0.22 µm pore size membrane filter into a sterile amber bottle or autoclave for 15 minutes at 121°C. Store at 2-8°C. Discard if solution becomes turbid.

b) STOCK MAGNESIUM CHLORIDE SOLUTION:

   Dissolve 38 g magnesium chloride (MgCl2) in 1L DI. Filter through a sterile 0.22 µm pore size membrane filter into a sterile amber bottle or autoclave for 15 minutes at 121°C. Store at 2-8°C. Discard if solution becomes turbid. No recommended final pH. Store at 2-8°C. Discard if solution becomes turbid.

c) BUFFERED DILUTION WATER:

   Add 1.25 mL stock PO4 buffer solution and 5mL stock MgCl2 solution to a 1 L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

   Dilution blanks: 10 mL in 20 x 150mm test tubes
   90 mL in milk dilution bottles

Autoclave 10 – 90 mL volumes at 121°C for 15 minutes. Test for sterility. Final volume should be 10 mL ± 2% and 90 mL ± 2%. Final pH should be 7.2 + 0.1 at 25°C. Store at room temperature for up to 1 month.

d) TETRATHIONATE BROTH BASE (DIFCO) SINGLE STRENGTH:

Formula (grams per litre):

i)	Proteose peptone	5.0 g
	Bacto bile salts	1.0 g
	Sodium thiosulfate	30.0 g
	Calcium carbonate	10.0 g
ii)	Iodine-iodide solution	
	Iodine crystals*	6.0 g
	Potassium Iodide	5.0 g
	Deionized water	20.0 mL

Grind iodine crystals with potassium iodide with a mortar and pestle. Dissolve in DI.

***Note**: Crystalline iodine is extremely volatile and corrosive. Do not use foil to cover beaker. Use fume hood and wear gloves when handling crystalline iodine.*

Suspend 46 g powdered medium in 1 L deionized water and heat to boiling. Cool below 60°C. Add 20 mL iodine-iodide solution per litre of tetrathionate broth base. Do not heat medium after adding iodine. Dispense 10 mL quantities into sterile 18 x 150 mm screw cap test tubes. Use medium the same day it is prepared. Final pH should be 8.4 ± 0.2 at 25°C or as per manufacturer's instructions. Test media as per manufacturer's instructions. Incubate at 35°C $\pm$ 2°C for 18-24 hours. Do not store after iodine-iodide solution has been added. Use media immediately. Media without iodine-iodide solution can be stored at 2-8°C for 3 months in screw cap tubes.

e) TETRATHIONATE BROTH BASE - DOUBLE STRENGTH:

Formula is as listed above.

Suspend 46 g in 500 mL deionized water and proceed as instructed above, using 40 mL iodine-iodide solution per litre. Dispense 20 mL quantities into sterile 25 x 150 mm screw cap test tubes. Final pH should be 8.4 ± 0.2 at 25°C or as per manufacturer's instructions. Test media as per manufacturer's instructions. Incubate at 35°C $\pm$ 2°C for 18-24 hours. Do not store after iodine-iodide solution has been added. Use media immediately. Media without iodine-iodide solution can be stored at 2-8°C for 3 months in screw cap tubes.

f) XLD AGAR, dehydrated (DIFCO):

Formula (grams per litre):

Bacto yeast extract	3.00 g
L-lysine	5.00 g
Xylose	3.75 g
Lactose	7.50 g
Saccharose	7.50 g
Sodium desoxycholate	2.50 g
Ferric ammonium citrate	0.80 g
Sodium thiosulfate	6.80 g
Sodium chloride	5.00 g
Bacto agar	15.0 g
Phenol red	0.08 g

Suspend 55g in 1 L deionized water and boil to dissolve completely. Avoid overheating. Do not autoclave. Cool to 45 to 50°C and dispense 17-20 mL into sterile 100 x 15mm petri plates. Final pH should be 7.4 ± 0.2 at 25°C or as per manufacturer's instructions. Test media as per manufacturer's instructions. Incubate at 35°C ± 2°C for 24 hours. Store at 2-8°C for 2 weeks. Protect from light.

g) BRAIN HEART INFUSION AGAR, dehydrated (BHIA), DIFCO:

Formula (grams per litre):

Calf brain infusion	200 g
Beef heart infusion	250 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Bacto agar	15.0 g

Suspend 52 g of powdered medium in 1 L deionized water and heat to boiling to dissolve completely. Sterilize in the autoclave for 15 minutes at 121°C. Dispense 17-20 mL into sterile 100 x 15 mm petri plates. Final pH should be 7.4 ± 0.2 at 25°C or as per manufacturer's instructions. Test media as per manufacturer's instructions. Incubate at 35°C ± 0.5°C for 24 hours.

h) NORMAL SALINE:

Sodium chloride	8.5 g
Deionized water	1.0 L

Dissolve NaCl in DI and autoclave 90mL aliquots in milk dilution bottles for 15 minutes at 121°C. Test for sterility. Final volume should 90 mL ± 2%. Final pH should be 7.2 + 0.1 at 25°C. Store at room temperature for up to 1 month.

i) 0.1% PEPTONE IN BUFFERED WATER:

Proteose peptone	1.0 g
Stock buffered water	1.0 L

Dissolve peptone in buffered water and heat to dissolve. Dispense in 90 mL aliquots in milk dilution bottles or in 10 mL aliquots in test tubes. Autoclave for 15 minutes at 121°C. Test for sterility. Final pH should be 7.2 + 0.2 at 25°C.

Procedure

Detailed, instructions for the MTF technique are provided in APHA 9221 B.

a) Set up test tube rack with a sequence of test tubes which includes at least 5 replicate tubes per sample volume and 3 dilutions.
b) Use double strength tetrathionate broth tubes for the initial sample volume of 10 mL per tube. Use single strength tetrathionate broth tubes for all subsequent sample volumes.
c) Prepare soil samples using one of the two following procedures:
Option 1: Weigh 10 g of as-received soil or sludge (not dried) into a sterile Stomacher® bag. Make sure there are no rocks or hard pieces of wood included. Add 90 mL 0.1% peptone in buffered water. Mix by "paddling" for 30 seconds in Stomacher® bag.
Option 2 (CSSS reference): Pass as-received soil sample (not dried) through a 2 mm mesh sieve and mix thoroughly. Weigh a 10 g soil portion into a dilution bottle containing 95 mL diluent. Sterile glass beads (~ 25 x 2 mm) may be added to aid in mixing. Cap the bottle, shake on a mechanical shaker for 10 minutes or shake by hand in 45° arc at least 50 times. Re-shake each sample vigorously prior to subsampling

d) Keep solids in suspension and dispense 10 mL aliquots into each of 5 double strength tetrathionate broth tubes, 1 mL aliquots into each of 5 single strength tetrathionate broth tubes, and decimal dilutions as required by serially diluting 1 mL aliquots in 10 mL buffered water blanks and inoculating single strength tetrathionate broth tubes.
e) Incubate tubes for 48 ± 3 hours at 35± 0.5°C.
f) Transfer a loopful of each culture to sections marked off on plates of XLD agar. Streak for isolated colonies. Incubate inverted agar plates at 35 ± 0.5°C for 24 ± 2 hours.
g) Pick any red colonies with black centers to purify on BHIA and incubate for 18-24 hours at 35 ± 0.5°C.
h) Mark off two ovals on a microscope slide and place a drop of saline and a drop of polyvalent "O" antiserum in either oval. Make a suspension of a well-isolated colony from BHIA first in the saline and then in the antiserum. Continue rocking the slide for 1-2 minutes to insure adequate mixing. Salmonella spp. will produce a strong positive agglutination in the polyvalent "O" antiserum with no agglutination in the saline control. If there is a questionable agglutination, specific antisera may be screened. Each Salmonella sp. will react with only one specific "O" antiserum in addition to the polyvalent antiserum. If the saline control agglutinates the organism is self-agglutinating or rough, and must be confirmed by biochemical reaction.
i) For biochemical confirmation of Salmonella spp. make a suspension in saline and proceed according to the instructions for inoculating and incubating an API 20E® strip.
j) Count only those tubes containing growth subsequently found to be Salmonella for calculations. Note that Proteus sp., Citrobacter sp. and Arizona sp. can sometimes resemble Salmonella sp. on XLD agar. Only Salmonella will agglutinate with polyvalent "O" antiserum. The biochemical profile of each genus is specific.

Note 1: All Salmonella are potentially pathogenic. All plates and suspensions must be handled in a biohazard hood. All refuse and spent media must be autoclaved for 30 minutes at 121°C before disposal.

Note 2: Use and handling instructions for control organisms, and quality control practice guidelines are not described in the manufacturer's instructions. Refer to APHA 9020 for guidance on these topics.

DATA ANALYSIS

Refer to a standard MPN index (e.g. Section 4.0 of the Laboratory Quality Assurance/Quality Control section of the Microbiology Examination Section of the laboratory manual) for guidance regarding calculation of bacterial density. Note that a minimum of 3 dilutions are required for this calculation.

Also refer to:

iii) APHA 9221 for details of MTF interpretation and MPN conversion.
iv) APHA 9260B for information on Salmonella quantitation tests.

Test results should be reported in units of CFU per gram of wet (as-received) soil.

Quality Control

Summary of QC Requirements		
QC Component	**Minimum Frequency**	**Minimum Data Quality Objectives**
Method Blank (MB)	One per batch (max 20 samples)	Less than reported DL
Lab Duplicates (DUP)	1 per batch (max 20 samples)	± 65% RPD
Positive & Negative Controls	One each per day per incubator	Expected reaction to confirm proper operation of incubator and performance of the test.
If DQOs are not met, repeat testing or report qualified test results.		

Method Blank: The Method Blank consists of the media / reagents used in sample preparation that proceeds through the same sample handling processes as test samples. It is recommended for the Method Blank to be prepared using a sample bottle/container.

Laboratory Duplicates: Sample duplicates are prepared when sufficient sample is received to subsample for laboratory duplicates. Homogenize the sample well prior to subsampling. Process both aliquots through the same sample handling processes as test samples.

Positive / Negative Controls: Two are recommended. Using both each day confirms that the test is performing as expected for all target and non-target organisms and that the incubator is operating as expected (reaches correct temperature at correct rate). Refer to APHA 9020 for more information.

Proofing of sample bottles, organisms, reagents, and supplies by lot is recommended to demonstrate sterility and performance prior to use. Refer to APHA 9020 for more information on recommended Quality Control practices for this test.

References

1. APHA 9221 (2014) Multiple-Tube Fermentation Technique for Members of the Coliform Group.
2. APHA 9260 (2007) Detection of Pathogenic Bacteria.
3. Canadian Society of Soil Science (CSSS), Soil Sampling and Methods of Analysis, 2nd Edition, edited by M.R. Carter, E.G. Gregorich.
4. APHA 9060 (2013) Samples.
5. APHA 9020 (2015) Quality Control.

Revision History

Feb 14, 1994	Publication in 1994 Lab Manual
Nov 14, 2002	SEAM codes replaced by EMS codes
Dec 20, 2019	Updated to BC Lab Manual Prescriptive Method format. Prescriptive nature of test is confirmed. APHA 9221 was revised in 2014. APHA 9260 was revised in 2007. APHA 9060 was revised in 2013. APHA 9020 was revised in 2015. Added simpler, alternative soil preparation option with CSSS reference. QC Section updated to include Method Blanks and Duplicate Samples. Changed sample storage temperature to <10°C as per APHA 9060 (2013).

Pseudomonas Aeruginosa in Fresh Water and Wastewater by Membrane Filtration (MF)

Parameter

Pseudomonas aeruginosa

Analytical Method and EMS codes

Membrane filter: **PSEU X022**

Scope

This method describes the selective isolation of Pseudomonas aeruginosa from water, wastewater, hot tubs and swimming pools. Non-turbid water samples are passed through a 0.45µm membrane filter which is placed on a selective agar (mPA-C) for up to 72 hours incubation at 41°C for growth of P. aeruginosa colonies. Colonies are purified on brain heart infusion agar (BHIA) and confirmed by API® Rapid NFT (Analytab Products.)

Principle

Pseudomonas aeruginosa belongs to the Family Pseudomonadaceae and are Gram negative, motile, oxidase positive rods that do not ferment glucose. P. aeruginosa can cause infections of the ear, upper respiratory tract, skin, and intestinal or genitourinary tract.

Sample Handling

The sample is collected in the field and submitted unpreserved in a sterilized water bacteriology bottle. Chlorinated water samples should be treated with a sodium thiosulfate (final concentration 0.01% w/v) to neutralize the bactericidal effect of chlorine. The sample should be kept at 4°C until analysis. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum volume required for analysis is 125mL.

Detection Limit

a) for duplicate 50ml samples the detection limit is 2 CFU/100ml.
b) for a total of 100ml the detection limit is 1 CFU/100ml.

Interferences

Excessive turbidity or particulate matter can interfere with filtration or cause clumping of the organism. Residual chlorine in chlorinated water must be neutralized with sodium thiosulfate at time of sampling.

Precision

There are no standard reference materials for Pseudomonas aeruginosa. Samples seeded with ATCC 27853 P. aeruginosa gave recoveries of 81 and 80% on mPA-C at 20 and 200 CFU/100mL and coefficients of variation of 15% and 9% respectively.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 41 ± 0.5°C temperature.
b) Sterile disposable serological pipettes, 1mL and 10mL.
c) Sterile 100mL glass graduated cylinders.
d) Sterile disposable petri dishes, 50mm x 12mm with tight fitting lids.
e) Sterile disposable petri dishes, 100m x 15mm.
f) mPA-C agar.
g) BHI agar.
h) Autoclave for steam sterilization of glassware and media.
i) Bunsen burner.
j) Platinum inoculation loops, 3mm diameter.
k) Glass filtration units, 250mL (Millipore® or equivalent), sterilized and wrapped in aluminum foil or kraft paper.

l) Presterilized membrane filters, 47mm diameter, white, grid marked, 0.45µm pore size, certified for bacteriology.
m) Vacuum source.
n) Vacuum flask and manifold to hold filtration units.
o) Smooth tipped forceps.
p) 95% ethanol, undenatured.
q) Gram staining reagents (available commercially from Difco).
r) Microscope slides and microscope with oil immersion lens.
s) API® Rapid NFT strips (available commercially from Analytab Products).
t) Oxidase reagent in sealed glass ampules (available from Difco or equivalent).
u) Stereobinocular microscope with cool white fluorescent light source.
v) Long wave UV light source, 366 nm (Wood's lamp).

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION.

Dissolve 34.0g of potassium dihydrogen phosphate (KH_2PO_4) in 500mL deionized water (DI). Adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1L with DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

b) STOCK MAGNESIUM CHLORIDE SOLUTION.

Dissolve 38g magnesium chloride ($MgCl_2$) in 1L DI. Filter through a sterile 0.22µm pore size membrane filter into a sterile amber bottle. Store at 4°C. Discard if solution becomes cloudy.

c) BUFFERED DILUTION WATER.

Add 1.25mL stock PO_4 buffer solution and 5mL stock $MgCl_2$ solution to a 1L volumetric flask and bring to volume with DI. Dispense into appropriate containers as follows:

Dilution blanks:	10mL in 20mm test tubes
	100mL in milk dilution bottles
Rinse water:	1500mL per 2L Erlenmeyer flask

Autoclave 10 - 100mL volumes at 121°C for 15 minutes; for larger volumes, increase the time as appropriate to achieve sterilization.

d) mPA-C AGAR (BBL)

Formula (grams per litre):

i)	Yeast extract	2.00 g
	L-Lysine HCl	5.00 g
	Sodium chloride	5.00 g
	Xylose	1.25 g
	Sucrose	1.25 g
	Lactose	1.25 g
	Phenol red	0.08 g
	Ferric ammonium citrate	0.80 g
	Sodium thiosulfate	5.00 g
	Kanamycin	0.008 g
	Nalidixic acid	0.037 g
	Agar	12.00 g
ii)	Cyclohexamide*	0.150 g

Suspend 35g powdered medium in 1L DI and heat to boiling. Boil for 1 minute to completely dissolve. Add cyclohexamide. Cool to 45-50°C and aseptically pour 4mL into sterile 50mm petri dishes. Final pH of medium is 7.2 at 25°C. May be stored for 1 week at 4°C.

***Note**: Cyclohexamide is a poison if swallowed or absorbed through skin. Wear gloves when weighing.*

e) BRAIN HEART INFUSION AGAR, dehydrated

Formula (grams per litre):

Calf brain infusion	200.0 g
Beef heart infusion	250.0 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Agar	15.0 g

Suspend 52g in 1L DI and boil to dissolve completely. Autoclave for 15 minutes at 121°C. Cool to 45 - 50°C and aseptically dispense 15 - 17mL into 100mm petri dishes.

f) ASPARAGINE BROTH
(Not currently available commercially.)

Formula (grams per litre):

Asparagine, DL	3.0 g
Anhydrous dipotassium hydrogen phosphate, K_2HPO_4	1.0 g
Magnesium sulfate, $MgSO_4.7H_2O$	0.5 g

Add ingredients to 1L DI water, and heat to dissolve completely. Adjust pH to 6.9 to 7.2. Dispense 10mL per 18mm test tube. Autoclave for 15 minutes at 121°C.

Procedure

a) Place a sterile membrane filter on a sterile filter base, grid side up and attach the funnel to the base of the filter unit.

b) Select a sample volume to produce 20 - 80 colonies on the membrane filter. Decimal dilutions are prepared in 10mL buffered water dilution blanks. Do not filter less than 10mL volumes.

c) Shake the sample bottle vigorously about 30 times and measure the desired volume of the sample into a sterile graduated cylinder for volumes of 20mL or more, or pipet directly into the funnel for volumes of 10mL or decimal dilutions of 10mL.

d) Filter the sample and rinse the sides of the funnel with 20 - 30mL sterile buffered water. Turn off the vacuum and remove the funnel from the filter base.

e) Aseptically remove the membrane filter from the filter base and place grid side up on the mPA-C agar. Reset if air bubbles are trapped under the filter.

f) Filter the samples in order of increasing sample concentration and/or volume, low bacterial density to high.

g) Prepare duplicate filters for each concentration or volume filtered. Incubate mPA-C agar plates for 24 - 48 hours at 41°C.

h) Pick any pinkish/greyish, low, spreading colonies to purify on BHIA or inoculate into asparagine broth and incubate for 18 - 24 hours at 35°C.

i) After incubation, pick a well-isolated colony from BHIA and Gram stain (heat fix smear on microscope slide, flood smear with Gram's crystal violet for 1 minute, rinse; flood with Gram's iodine for 1 minute, rinse; decolorize with Gram's acetone alcohol; counterstain with Gram's safranine for 30 sec). Examine with oil immersion microscope. P. aeruginosa are Gram negative rods, 1-2µm x 0.75-1µm in size.

j) Wet a filter pad with oxidase reagent and smear colony on filter with platinum loop. P. aeruginosa is oxidase positive.

k) Suspend isolated colony in 5mL sterile saline for inoculation into API® Rapid NFT strip and follow directions provided in kit.

l) Alternatively, shine long wave UV light on broth cultures grown in asparagine broth. P. aeruginosa will fluoresce.

m) Colonies conforming to the description given for P. aeruginosa are counted. Pinkish-grey, low, spreading colonies are counted using a stereobinocular microscope illuminated with cool white fluorescent light. Yellow colonies or colonies producing a yellow reaction in the agar are negative. Choose a plate with 20-80 colonies for counting.

Data Analysis

a) Calculate the bacterial density using the following formula:

$$\text{P. aeruginosa (*CFU/100mL)} = \frac{\text{Number of P. aeruginosa counted} \times 100}{\text{Volume of sample filtered}}$$

*Colony forming units

b) Counts on plates with less than 20 colonies are noted as "estimated" counts.

c) Plates with no colonies are reported as less than the calculated value/ 100mL based on the single largest volume filtered. Values are routinely reported as <2 CFU/100mL for largest single volume of 50mL. However, if there are no colonies on either of the 50mL duplicate plates the volume may be taken as 100mL and reported as <1 CFU/100mL.

Quality Control

95% confidence limits for membrane plate counts are calculated as follows:

Counts between 1 - 10 | Counts between 11 - 20

Counts	Lower	Upper	Counts	Lower	Upper
1	0.0	3.7	11	5.4	19.7
2	0.025	5.6	12	6.2	21.0
3	0.24	7.2	13	6.9	22.3
4	1.1	10.2	14	7.7	23.5
5	1.6	11.7	15	8.4	24.8
6	2.2	13.1	16	9.4	26.0
7	2.8	14.4	17	9.9	27.2
8	3.5	15.8	18	10.7	28.4
9	4.1	17.1	19	11.5	29.6
10	4.8	18.4	20	12.2	30.8

For counts greater than 20 use the following formulae:
upper limit = $C + 2\sqrt{C}$
lower limit = $C - 2\sqrt{C}$
Where C = number of colonies counted.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9213.

b) Dutka, B. "Pseudomonas aeruginosa as Indicator Pathogen." Membrane Filtration: Applications, Techniques and Problems. Bernard Dutka (Ed.) Marcel Dekker, Inc. New York, 1981.

c) Monograph. Manual of BBL Products and Laboratory Procedures, 6th Edition, Power, D.A., and McCuen, P.J. (Eds) Cockysville, MD, 1988.

Revision History

February 14, 1994: Publication in 1994 Laboratory Manual.
November 14, 2002: SEAM Codes replaced by EMS codes.