Multiple-Tube Fermentation for Thermotolerant Coliform Bacteria in Bivalve Molluscan Shellfish - 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 Enterobacteriaceae. 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)**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Approx. MDL</th>
<th>EMS Analyte / Method Codes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermotolerant (Fecal) Coliforms</td>
<td>18 CFU /100 g</td>
<td>0450 / X390</td>
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</table>

*Refer to EMS Parameter Dictionary on the ministry 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.
o) Sterile buffered dilution water.

Reagents

a) **STOCK PHOSPHATE (PO₄) BUFFER SOLUTION:**

Dissolve 34.0 g of potassium dihydrogen phosphate (KH₂PO₄) 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 MgSO₄ •7H₂O 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:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Tryptose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>5.00 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.75 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.75 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.00 g</td>
</tr>
<tr>
<td>Sodium lauryl sulfate</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.00 L</td>
</tr>
</tbody>
</table>

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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptose or trypticase</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Bile salts No.3</td>
<td>1.5 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>4.0 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 L</td>
</tr>
</tbody>
</table>

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.

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

### Quality Control

<table>
<thead>
<tr>
<th>Summary of QC Requirements</th>
<th>Minimum Frequency</th>
<th>Minimum Data Quality Objectives</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC Component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method Blank (MB)</td>
<td>One per batch (max 20 samples)</td>
<td>Less than reported DL</td>
</tr>
<tr>
<td>Lab Duplicates (DUP)</td>
<td>1 per batch (max 20 samples)</td>
<td>± 65% RPD</td>
</tr>
<tr>
<td>Positive &amp; Negative Controls</td>
<td>One each per day per incubator</td>
<td>Expected reaction to confirm proper operation of incubator and performance of the test.</td>
</tr>
</tbody>
</table>

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**


**Revision History**

- **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.