Section E

MICROBIOLOGICAL EXAMINATION

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INTRODUCTION

1.0 General

The analytical procedures following section 6.0 provide means of assessing the sanitary quality of waters and wastewaters and are meant to furnish information relating to the degree and possible sources of contamination with septic waste. Included in the analytical section are methods for the key parameters used for compliance monitoring and evaluation of water resources. The procedures have been written in a stepwise format so that the methods can be used as either a bench manual or reference documents.

2.0 Intralaboratory Quality Assurance

Sections 1.0 through 5.0 provide detailed guidance on quality control practices, specific to microbiological examination, that should be performed to ensure the production of accurate and valid data. Due to the lack of availability of objective, external quality assurance reference materials, the rigid control of all factors that could bias the analytical results assumes increased importance. These factors include:

- Sampling; including sampling equipment and containers.
- Sample shipment and storage.
- Laboratory facilities.
- Personnel.
- Instrument calibration and maintenance.
- Equipment, supplies and media.
- Analytical protocols.
- Record keeping.

Standard Methods, (Standard Methods for the Examination of Water and Wastewater, APHA, AWWA & WEF, 18th Edition, 1992, Section 9020), also contains an exhaustive dissertation on the subject, including a comprehensive checklist for all variables.

3.0 Interlaboratory Quality Assurance

Participation in interlaboratory quality assurance activities is encouraged (mandatory for laboratories providing data to the Ministry's SEAM data base). Available programs include the B.C. Laboratory Registration Interlaboratory Studies administered under the British Columbia Environmental Data Quality Assurance Regulation and the CAEAL interlaboratory program which is administered by the Canadian Association of Environmental Analytical Laboratories.

MICROBIOLOGICAL QUALITY ASSURANCE/QUALITY CONTROL

1.0 Reagent Water

1.1 <u>Distilled Water</u>

Distilled water used for the preparation of media should be free of inorganic and organic substances, either toxic or nutritive, that could influence the survival or growth of bacteria and viruses. Factors affecting the water quality include the design of the distillation apparatus, sources of raw water, use of carbon filter, storage chamber for reserve supply, temperature of the stored supply and length of storage prior to use. These factors may influence the extent of bacterial and chemical contamination, and often the pH.

Maintenance Procedure		Frequency
1.	Drain and clean boiler and condenser	Monthly
2.	Drain and clean (acid wash) distilled water reservoir, boiler and condenser	Quarterly
3.	Chemical testing	Monthly
4.	Water quality test (see 1.2)	Annually
5.	Standard plate count (optional)	

***Note**: the water quality test should be performed on raw water, distilled water (fresh) and the rinse cycle from the glassware washer.*

Test	Limit
Conductivity	>0 . 5 megohms resistance or <2 µmhos/cm at 25°C
pH	5.5 - 7.5
Total organic carbon	<1.0mg/L
Heavy metals, total	<0.5 mg/L
Ammonia/organic nitrogen	≤1.0 mg/L
Total chlorine residual	<detection limit
Bacteriological tests:	
Standard plate count	<1000 colonies/mL
Water quality test	0.8 - 3.0 ratio
Use test (statistics)	Students t ≤2.78

1.2 Water Quality Test

This procedure is a test for the determination of toxic or stimulator effects of distilled or deionized water on bacteria. It is based on the growth of Enterobacter aerogenes in a chemically defined medium. Reduction of 20% or more in bacterial population compared to a control is judged toxic. Increased growth greater than 300% is called stimulator.

The full procedure is described in the following texts:

i) Standard Methods for the Examination of Water and Wastewater, 18th edition (1992).

ii) Microbiological Methods for Monitoring the Environment. Water and Wastes. EPA-600/8-78-017 (1978).

1.3 Deionized Water

Deionized water may be substituted for distilled water if the latter is not suitable or by specific request for special media.

Maintenance Procedure		Frequency
1.	Check Filter Units	Quarterly
2.	Chemical testing	Quarterly
3.	Standard Plate Count	Quarterly*

*Test should be completed prior to the regeneration of the filter units.

1.4 **Inhibitory Residue Test**

Test for inhibitory residues on glassware annually. Certain wetting agents or detergents used in washing may contain bacteriostatic or inhibiting substances requiring exhaustive rinsing to remove all traces of residual bacteriostatic action. Test all new supplies of detergent using the following procedure:

1. Wash and rinse six glass petri dishes according to usual laboratory practice and designate as Group A.

2. Wash six glass petri dishes as above, rinse 12 times with reagent grade DI and designate as Group B.

3. Rinse six petri dishes with detergent wash water (in use concentration), dry without further rinsing, and designate as group C.

4. Autoclave petri dishes.

5. Add no more than 1mL of a culture of Enterobacter aerogenes known to contain 50 - 150 colony forming units (CFU) to dishes in Groups A - C and proceed as for a pour plate heterotrophic plate count (see relevant section, Heterotrophic Plate Count) using Plate Count Agar, R2A, or NWRI agar. Preliminary testing may be necessary to obtain the specified count range.

6. Differences in averaged counts on plates in Groups A - C should be less than 15% if there are no toxic or inhibitory effects. Differences in averaged counts of less than 15% between Groups A and B and greater than 15% between Groups A and C indicates that the cleaning detergent has inhibitory properties that are eliminated during routine washing.

2.0 Equipment

2.1 **pH Meter**

	Maintenance Procedure	Frequency
1.	Compensate for temperature	each use
2.	Date standard buffer solutions when first opened and discard if pH is more than ±0.1 pH units from manufacturer's stated value or is contaminated with microorganisms	as required
3.	Standardize with at least one standard buffer (usually pH 7.0)(Do not re-use buffer solution)	before each use
4.	Check buffer standard reading against another pH meter	monthly

2.2 **Balance**

Maintenance Procedure		Frequency
1.	Check balance with a set of certified weights	monthly
2.	Wipe balance after each use	each use
3.	Calibration	annually

***Note**: For general media preparation, balance must have a sensitivity of 0.1g at a load of 150g.*

2.3 **Autoclave/Sterilizer**

Maintenance Procedure		Frequency
1.	Chart record of sterilization cycle	record for each cycle date and keep records for 2 years.
2.	Check operating temperature with a maximum/minimum thermometer	monthly
3.	Test performance with spore suspension (Use Oxoid spore strips BR23 and spore strip broth CM763)	monthly or as required by
4.	Preventive maintenance inspection	annually
5.	Sterility test tape	each load
6.	Drain and rinse boiler and clean	chamber if applicable weekly
7.	Check door seal	semi-annually

2.4 **Glassware Washer**

Maintenance Procedure		Frequency
1.	Clean washer tubes and drain reservoir	if applicable at least daily
2.	Maintain reservoir wash water at 70 - 80°C	ongoing
3.	Clean reservoir if applicable	weekly during use
4.	Check hoses, drive chain, pumps	quarterly
5.	Inhibitory residue test	annually
6.	Chemical testing	quarterly

2.5 **Hot Air Oven**

	Maintenance Procedure	Frequency
1.	Test performance with spore strips	quarterly or as required
2.	Monitor temperature with a thermometer in the 160-180°C range (insert thermometer bulb up to the immersion mark into a sand-filled graduated cylinder or beaker)	accurate ongoing

2.6 **Incubators**

	Maintenance Procedure	Frequency
1.	Check temperature record and thermometer	daily during use
2.	Drain and clean water baths	monthly
3.	Measure temperature in dry incubators	daily

***Note**: If partially submersible glass thermometers are used, bulb and stem must be immersed in water or glycerol to mark on the stem*

2.7 **Automatic Pipettor**

	Maintenance Procedure	Frequency
1.	Check accuracy of dispensation using a graduated cylinder at the start of each volume change and periodically throughout extended runs.	ongoing
2.	After dispensing each type of medium, pass a large volume of distilled water through the dispenser to remove traces of agar or medium.	ongoing
3.	At the end of the work day rinse thoroughly with 70% ethanol, break down into unit parts and wash well.	ongoing

2.8 **Refrigerators**

	Maintenance Procedure	Frequency
1.	Check temperature (4°C)	daily
2.	Clean and disinfect	monthly
3.	Date stamp and identify all material	ongoing
4.	Discard outdated material	quarterly

2.9 Thermometers and Recording Devices

Maintenance Procedure	Frequency
1. Check the accuracy of thermometers and temperature recording instruments against an NIST certified thermometer. Graduations should not exceed 0.1°C for water bath (elevated temperature) incubation and 0.5°C for dry air incubation (35°C). Record calibration correction on thermometer or on outside of incubator, waterbath or refrigerator.	annually

2.10 Summary of Equipment Maintenance Procedures and Frequencies

Frequency	Activity
Daily or before each use	1. Clean glassware washer tubes, rinse and drain reservoir, if applicable 2. Standardize pH meter and compensate for temperature 3. Change temperature/pressure recording chart for autoclave (as needed) 4. Use sterile tape for each autoclave load 5. Measure temperature in incubators, ovens, refrigerators 6. Clean automatic pipettor
Weekly	1. Drain and clean washer reservoir, depending on use 2. Drain and rinse autoclave boiler and clean chamber
Monthly	1. Drain and clean still boiler and condenser 2. Check pH buffer reading against another pH meter 3. Check balance with set of certified weights 4. Check autoclave operating temperature with a maximum/minimum thermometer 5. Test sterilization performance of autoclave with spore suspension 6. Drain and clean incubator waterbaths 7. Clean and disinfect refrigerators 8. Chemical testing of distilled water
Quarterly	1. Drain and clean (acid wash) distilled water reservoir, boiler and condenser 2. Standard plate count on distilled water 3. Discard outdated material from refrigerators 4. Chemical testing for glassware washer 5. Check hoses, drive chain and pumps of glassware washer 6. Check filter units on deionizer 7. Chemical testing and standard plate count on deionized water
Semi-annually	1. Check autoclave door seal

Annually	1. Water quality test on distilled water 2. Balance calibration 3. Preventive maintenance inspection of autoclave 4. Inhibitory residue test for glassware washer 5. Check accuracy of thermometers

3.0 Media Preparation and Storage Requirements

1. Measure pH of media prior to sterilization and adjust appropriately with 1N NaOH or 1N HCL.

2. Measure pH of media after sterilization. ***Note*** pH may shift 0.1 to 0.2 pH units lower during sterilization and allowance must be made for this shift.

3. Store prepared media at ambient temperature or in a cold room away from direct sunlight.

4. Unless screw cap tubes are used, discard media after two weeks.

5. Discard dehydrated media which are more than 5 years old or which have become caked or hardened.

6. Conduct quality control checks of new media lots using control cultures.

4.0 Analysis Quality Control

1. Replication. Conduct duplicate analyses on 10% of the known positive samples.

2. Controls. Include 1 pure culture positive control, 1 pure culture negative control and 1 sterile control for each parameter tested. For continuous, routine analysis this should be done monthly. For occasional or short-term intensive programs, controls should be used for each program. Table 5 provides appropriate control organisms for specific tests.

3. Colony Counting. The same MF and SPC plates should be counted by two or more analysts monthly or as dictated by laboratory workload.

4. MF Verification. Five percent of all MF analyses performed should be verified. Verification techniques are described in Standard Methods. For the MPN test, five percent of the positive samples and/or a minimum of 1 sample per test run should be verified.

5. Computing and Recording of MPN. Record the number of positive findings of coliforms in each dilution and compute in terms of the MPN. The MPN values, for a variety of planting series and results, are given in Tables 1, 2 and 3. Included in these tables are the 95% confidence limits for each MPN value determined. When the series of dilutions is different from that in the table, select the MPN value from Table 3 for the combination of positive tubes and calculate according to the following formula:

$$\text{MPN value (from the table)} \times \frac{10}{\text{largest volume tested}} = \text{MPN/100 mL}$$

6. Documentation

95% confidence limits for MPN are included in the standard MPN index.

- Analyst signs off work sheet.
- Date of preparation and lot number of medium is recorded.
- Temperatures of incubators and water bath are recorded weekly.

Table 1.

MPN Index and 95% Confidence Limits for Various Combinations of Positive and Negative Results when Five 10 mL Portions are Used

No. of Tubes Giving Positive Reaction Out of 5 of 10 mL Each	MPN Index 100 mL	95% Confidence Limits (Approximate) Lower	Upper
0	<2.2	0	6.0
1	2.2	0.1	12.6
2	5.1	0.5	19.2
3	9.2	1.6	29.4
4	16.0	3.3	52.9
5	>16.0	8.0	Infinite

Table 2.

MPN Index and 95% Confidence Limits for Various Combinations of Positive and Negative Results when Ten 10 mL Portions are Used

No. of Tubes Giving Positive Reaction Out of 10 of 10 mL Each	MPN Index 100 mL	95% Confidence Limits (Approximate) Lower	Upper
0	<1.1	0	3.0
1	1.1	0.03	5.9
2	2.2	0.26	8.1
3	3.6	0.69	10.6
4	5.1	1.3	13.4
5	6.9	2.11	6.8
6	9.2	3.1	21.1
7	12.0	4.3	27.1
8	16.1	5.9	36.8
9	23.0	8.1	59.5
10	>23.0	13.5	Infinite

Table 3.

MPN Index And 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes Are Used Per Dilution (10 mL, 1.0 mL, 0.1 mL)

Combination of Positives	MPN Index 100 mL	95% Confidence Limits Lower	Upper		Combination of Positives	MPN Index 100 mL	95% Confidence Limits Lower	Upper
0-0-0	<2	-	-	*	4-2-0	22	9.0	56
0-0-1	2	1.0	10	*	4-2-1	26	12	65
0-1-0	2	1.0	10	*	4-3-0	27	12	67
0-2-0	4	1.0	13	*	4-3-1	33	15	77
				*	4-4-0	34	16	80
1-0-0	2	1.0	11	*	5-0-0	23	9.0	86
1-0-1	4	1.0	15	*	5-0-1	30	10	110
1-1-0	4	1.0	15	*	5-0-2	40	20	140
1-1-1	6	2.0	18	*	5-1-0	30	10	120
1-2-0	6	2.0	18	*	5-1-1	50	20	150
				*	5-1-2	60	30	180
2-0-0	4	1.0	17	*	5-2-0	50	20	170
2-0-1	7	2.0	20	*	5-2-1	70	30	210
2-1-0	7	2.0	21	*	5-2-2	90	40	250
2-1-1	9	3.0	24	*	5-3-0	80	30	250
2-2-0	9	3.0	25	*	5-3-1	110	40	300
2-3-0	12	5.0	29	*	5-3-2	140	60	360
3-0-0	8	3.0	24	*	5-3-3	170	80	410
3-0-1	11	4.0	29	*	5-4-0	130	50	390
3-1-0	11	4.0	29	*	5-4-1	170	70	480
3-1-1	14	6.0	35	*	5-4-2	220	100	580
3-2-0	14	6.0	35	*	5-4-3	280	120	690
3-2-1	17	7.0	40	*	5-4-4	350	160	820
4-0-0	13	5.0	38	*	5-5-0	240	100	940
4-0-1	17	7.0	45	*	5-5-1	300	100	1300
4-1-0	17	7.0	46	*	5-5-2	500	200	2000
4-1-1	21	9.0	55	*	5-5-3	900	300	2900
4-1-2	26	12	63	*	5-5-4	1600	600	5300
				*	5-5-5	>1600	-	-

Table 4.

Control Culture Nomenclature

Genus	Species	Abbreviation
Citrobacter	freundii	C. freundii
Clostridium	perfringens	C. perfringens
Enterobacter	aerogenes	E. aerogenes
Enterobacter	cloacae	E. cloacae
Escherichia	coli	E. coli
Klebsiella	pneumoniae	K. pneumoniae
Proteus	mirabilis	P. mirabilis
Proteus	vulgaris	P. vulgaris
Pseudomonas	aeruginosa	P. aeruginosa
Salmonella	typhimurium	S. typhimurium
Salmonella	typhosa	S. typhosa
Serratia	marcescens	S. marcescens
Shigella	flexneri	S. flexneri
Staphylococcus	aureus	S. aureus
Streptococcus	fecalis	S. fecalis
Streptococcus	fecalis var. zymogenes	S. fecalis var. zymogenes
Streptococcus	fecalis var. liquefaciens	S. fecalis var. liquefaciens
Streptococcus	fecium	S. fecium
Streptococcus	mitis-salivarius	S. mitis-salivarius
Streptococcus	pyogenes	S. pyogenes

Table 5.
Quality Control of Media and Biochemical Tests

Medium	Control Cultures	Expected Results
M-Endo MF Broth or Agar	E. coli	Golden green metallic sheen
	E. aerogenes	Golden green metallic sheen
	Achromobacter sp.	Red colonies
	Pseudomonas sp.	Red colonies
	Salmonella sp.	Red colonies if medium overheated
M-FC Broth or Agar	E. coli	Blue colonies
	K. pneumoniae	Blue colonies
	E. aerogenes	No growth
Brilliant Green Bile Lactose Broth	E. coli	Growth with gas
	E. aerogenes	Growth with gas
	C. freundii	Growth with gas
	S. aureus	No growth
Lauryl Tryptose Broth	E. coli	Growth with gas
	E. aerogenes	Growth with gas
	S. typhimurium	Marked to complete inhibition
	S. aureus	Marked to complete inhibition
Levine's Eosin Methylene Blue Agar	E. coli	Nucleated black colonies with golden green metallic sheen
	E. aerogenes	Pink colonies with dark centres
	C. freundii	Yellow/beige colonies
	Salmonella sp.	Yellow/beige colonies
	Klebsiella sp.	Large brown mucoid colonies
Xylose Lysine Desoxycholate Agar (XLD)	Salmonella sp.	Red colonies, to red with black centres
	Klebsiella sp.	Yellow colonies
	E. coli	Yellow colonies
	E. aerogenes	Yellow colonies
Bismuth Sulfite Agar	S. typhosa	Black colony with black or brownish black zone, with or without sheen
	Other Salmonella sp.	Raised green colonies
	Coliforms	Green colonies
Brilliant Green Agar	Salmonella sp.	Pink-white opaque colonies surrounded by brilliant red zone
	E. coli	Inhibition or yellow green colonies
	P. vulgaris	Marked to complete inhibition or red colonies.

Medium	Control Cultures	Expected Results
KF Streptococcus Agar	S. fecalis	Pink to red colonies
	S. pyogenes	No growth
	S. aureus	No growth
	E. coli	No growth
PSE Agar	S. fecalis	Black colonies
	E. coli	No growth
	S. aureus	No growth
BHI Broth at pH 9.6	S. fecalis	Positive: growth
	S. mitis-salivarius	Negative: no growth
BHI Broth with 6.5% NaCl	S. fecalis	Positive: growth
	S. mitis-salivarius	Negative: no growth
Arginine Dehydrolase (Moeller's medium)	S. typhimurium	Positive: alkaline reaction, reddish violet colour
	S. flexneri	Negative: acid reaction, yellow
Lysine Decarboxylase (Moeller's medium)	S. typhimurium	Positive: alkaline reaction, reddish violet colour
	S. flexneri	Negative: acid reaction, yellow
Ornithine Decarboxylase (Moeller's medium)	S. typhimurium	Positive: alkaline reaction, reddish violet colour
	S. flexerni	Negative: acid reaction, yellow
Indole Production (Tryptophane Broth)	E. coli	Positive: red colour
	Salmonella sp.	Negative: orange/yellow colour
	E. aerogenes	Negative: orange/yellow colour
Methyl Red (Buffered Peptone Glucose Broth)	E. coli	Positive: red colour
	E. aerogenes	Negative: no change
Voges-Proskauer (Buffered Peptone Glucose Broth)	E. aerogenes	Positive: pink colour
	E. coli	Negative: no colour change
Citrate Utilization (Simmons Citrate Broth)	E. aerogenes	Positive: growth, change to blue colour
	E. coli	Negative: no colour change, no growth
Urease Production (Christensen's Urea Agar)	P. mirabilis	Positive: colour change, pink to red
	Salmonella sp.	Negative: no colour change

Medium	Control Cultures	Expected Results
Catalase (BHI agar slant)	S. aureus S. fecalis	Positive: bubbles Negative: no bubbles
Cytochrome Oxidase (Alpha-napthol and para-aminodimethylanilineoxalate)	P. aeruginosa E. coli S. aureus	Positive: blue colour Negative: no change Negative: no change
Phenylalanine Deaminase (Phenylalanine Agar)	P. mirabilis Salmonella sp. E. coli	Positive: green colour Negative: no colour change Negative: no colour change
Malonate Utilization (Malonate Broth)	K. pneumoniae E. coli change	Positive: blue colour Negative: no growth or colour
Milk, Methylene Blue, 0.1%	S. fecalis Group Q Streptococci S. mitis-salivarius P. aeruginosa C. perfringens	Positive: reduction of methylene blue Negative: no growth Negative: no growth Peptonization and digestion Acid, coagulation and gas
Nitrate Reduction (Potassium Nitrate Broth)	E. coli P. aeruginosa	Positive: red colour change Negative: no colour change, gas production
2,3,5-Triphenyl Tetrazolium Chloride in TG Agar	S. fecalis S. fecium	Positive: reduction of TTC (red colour) Negative: no color change
Tellurite Agar	S. fecalis S. fecium	Growth No Growth
Beta-Hemolysis in Blood Agar	S. fecalis var. zymogenes S. fecalis	Positive: lysis of red blood cells Negative: no lysis of red blood

Hydrogen Sulfide	Slant	Butt	H_2S Production	
(Triple Sugar Iron Agar)	E. coli	A	A G	-
	P. vulgaris	A	A G	+
	S. typhimurium	K	A G	+

Lysine	Slant	Butt		H_2S Production
(Lysine Iron Agar)	S. typhimurium	K	K	+
	S. flexneri	K	A	+

Medium	Control Cultures	Expected Results
Gelatin Liquefaction at 20°C (Nutrient Gelatin)	S. marcescens	Positive: liquefaction
	S. fecalis var. liquefaciens	Positive: liquefaction
	S. fecalis	Negative: no liquefaction
	C. perfringens	Positive: liquefaction
	E. coli	Negative: no liquefaction
API	K. pneumoniae	see API Instruction Manual
	E. cloacae	
	P. vulgaris	
	P. aeruginosa	

Adapted from "Microbiological Methods for Monitoring the Environment, Water and Wastes" U.S.EPA (1978)

5.0 Laboratory Records

5.1 Media Usage and Inventories

1. All media received in the laboratory will be numbered and date stamped. A record of the media inventory will include the following information: date received; date opened; date finished; manufacturer; control/lot number; bottle number (internally assigned).

2. A record of prepared media will be maintained with the following information: media type; bottle number; preparation date and purpose; quantity prepared; pH (pre- and post-sterilization); control/lot number.

3. All prepared media will be labelled with the following information: media type; preparation date; bottle or batch number.

5.2 Maintenance Schedules

1. Maintenance and QA/QC schedules will be developed for each piece of equipment and results will be recorded as appropriate.

5.3 Temperature Records

1. Temperature records from recording thermometers will be date stamped and kept for a two year period for the following equipment: autoclave, waterbaths, dry air incubators.

5.4 Analytical Test Results

1. Suitable bench sheets will be developed to record the results of QA/QC testing and to permit tracing to the media batch/lot numbers.

2. A summary of test types and number of analyses will be prepared monthly.

6.0 Laboratory Safety Information

The following elements of a laboratory safety program have been excerpted from "Microbiological Methods for Monitoring the Environment, Water and Wastes" *published by the U. S. Environmental Protection Agency*.

6.1 Administrative Considerations

1. Laboratory has a formal documented safety program.
2. Each worker has a copy of the safety program.
3. Employees are aware of procedures for reporting accidents and unsafe conditions.
4. New employees are instructed on laboratory safety.
5. Joint supervisor-employee safety committee has been established to identify potential laboratory hazards.
6. Records are maintained of accidents and consequences.
7. Name and phone number of the supervisor and an alternate are posted at door of the laboratories so they may be contacted in case of an emergency.
8. At least one permanent employee has attended appropriate first aid courses.
9. Emergency telephone numbers for fire, ambulance, health centres, and poison control centre are placed in a conspicuous location near the telephone.
10. Employees know the location of first aid supplies.
11. Emergency first aid charts, and hazardous agents charts are posted in the laboratory.
12. Fire evacuation plan is established for the laboratory and is posted in a conspicuous location.

6.2 Personal Conduct

1. Personal clothing is stored outside of the microbiology laboratory.
2. Lab coats and street clothes are kept in separate lockers.
3. Lab coats are worn at all times in the laboratory. Surgical gloves are worn when handling contaminated samples (eg. sewage).
4. Germicidal soap or medicated surgical sponges are available for employees' use.
5. Consumption and preparation of food and beverages are not permitted in the laboratory.
6. Smoking or chewing gum are not permitted in the laboratory.
7. Food or drinks are not to be stored in laboratory refrigerators.
8. Reading materials are not kept in the laboratory.

9. Lab coats are not to be worn outside the laboratory.

10. Employees who have cuts, abrasions, etc., on face, hands, arms, etc., do not work with infectious agents.

11. Safety glasses are worn when working with toxic or corrosive agents and during exposure to UV radiation.

6.3 Laboratory Equipment

1. Bulb or mechanical device is used for all pipetting.

2. Pipets are immersed in disinfectant after use.

3. Benches are maintained in a clear and uncluttered condition.

4. Centrifuge cups and rubber cushions are in good condition.

5. A suitable disinfectant is available for immediate use.

6. Blender is used with sealed container assembly.

7. Microscopes, colony counters, etc., are kept out of the work area.

8. Water baths are clean and free of growth and deposits.

9. Employees are instructed in the operation of the autoclave and operating instructions are posted near the autoclave.

10. Autoclaves, hot air sterilizing ovens, water distilling equipment, and centrifuges are checked routinely for safe operation.

11. Broken, chipped or scratched glassware should not be used.

12. Broken glass is discarded in designated containers.

13. Electrical circuits are protected against overload with circuit breakers or ground-fault breakers.

14. Power cords, control switches and thermostats are in good working order.

15. Water taps are protected against back-siphoning.

6.4 Disinfection/Sterilization

1. Proper disinfectant is used routinely to disinfect table tops and carts before and after laboratory work.

2. Receptacles of contaminated items are marked.

3. Performance checks of autoclaves, gas sterilizers and hot air ovens are conducted with the use of spore strips, spore ampules, indicators, etc.

6.5 Biohazard Control

1. Biohazard tags or signs are posted in hazardous areas.
2. Safety cabinets of the appropriate type and class are provided.
3. Lab personnel are vaccinated for typhoid fever, tetanus and polio.
4. Floors are wet-mopped weekly with a disinfectant solution.
5. Personnel are trained in the proper procedures for handling lyophilized cultures when used.

6.6 General Handling and Storage of Chemicals and Gases

1. Containers of reagents and chemicals are labelled properly, according to WHMIS regulations.
2. Flammable solvents are stored in an approved storage cabinet or well-ventilated area away from burners, hot plates, etc.
3. Bottle carriers are provided for hazardous substances.
4. Gas cylinders are securely clamped to a firm support.
5. Toxic chemicals are clearly marked *poison or toxic*.
6. Material safety data sheets (MSDS) are available for all chemicals and gases in use or storage.

6.7 Emergency Precautions

1. Foam and carbon dioxide fire extinguishers are installed within easy access to laboratory and are properly maintained.
2. Eye wash stations, showers, oxygen respirators and fire blankets are available within easy access.
3. Fire exits are marked clearly.
4. First aid kits are available and in good condition.
5. At least one full-time employee is trained in first aid.
6. Source of medical assistance is available and known to employees.

Standard Plate Count by Membrane Filtration

Parameter

Standard Plate Count

Analytical Method And EMS Code

Membrane Filter: SPCN X385

Scope

This method describes the non-selective isolation of viable heterotrophic bacteria in aqueous samples. Bacterial colonies may arise from pairs, chains, clusters, or single cells, all of which are included in the term Colony Forming Units (CFU). No differentiation is made of colony types. If required, heterotrophic plates may be used for subsequent bacterial taxonomy. Decimal dilutions of sample are passed through a 0.45 µm membrane filter which is then placed on a nutrient agar for 48 hour incubation at 35°C for the growth of bacterial colonies.

Principle

The heterotrophic (standard) plate count provides an estimate of the number of viable heterotrophic bacteria in an aqueous sample.

Sample Handling

Field samples are submitted unfiltered and unpreserved but should contain sodium thiosulphate (0.01% v/v) if the sample is chlorinated. The sample should be collected in a sterilized water bacteriology bottle (1L), and kept at 4°C until analysis. Variations in temperature are to be avoided. Minimum volume required for analysis is 125mL. Analysis must be initiated within 24 hours of collection (the recommended maximum elapsed time between collection and examination of samples is 8 hours).

Range

0.01 - 100,000 CFU/mL

Detection Limit

0.01 CFU/mL or 1 CFU/100mL

Interferences

Excessive turbidity or particulate matter can interfere with filtration or cause clumping of the organisms. Interaction of mixed bacterial populations may be inhibitory for some bacteria.

Precision

There is no standard reference material for heterotrophic plate counts.

Apparatus and Materials

a) Incubator which is capable of maintaining a stable temperature (35 ± 0.2°C).
b) Sterile serological disposable pipettes, 1mL and 10mL.
c) Sterile 100mL glass graduated cylinders.
d) Sterile disposable petri dishes, 50mm x 12mm with tight fitting lids.
e) Autoclave for steam sterilization of glassware and media.
f) Bunsen burner.
g) Platinum inoculation loops, 3mm diameter.
h) 250mL glass filtration units (Millipore or equilivent), sterilized and wrapped in aluminum foil or kraft paper.
i) Presterilized membrane filters, 47mm diameter, white, grid marked, 0.45µm pore size, certified for bacteriology.
j) Vacuum source.
k) Vacuum flask and manifold to hold filtration units.
l) Smooth tipped forceps.

m) 95% ethanol.
n) Stereobinocular microscope with cool white fluorescent light source.

Reagents

a) STOCK PHOSPHATE (PO_4) BUFFER SOLUTION.

Dissolve 34.0g of potassium dihydrogen phosphate ($KH_2\ PO_4$) 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) R2A AGAR (DIFCO)

Proteose peptone No.3	0.50 g
Bacto yeast extract	0.50 g
Bacto dextrose	0.50 g
Sodium pyruvate	0.30 g
Casamino acids	0.50 g
Soluble starch	0.50 g
Dipotassium hydrogen phosphate	0.30 g
Magnesium sulfate heptahydrate	0.05 g
Bacto agar	15.00 g

Suspend ingredients in 1L DI. Boil to dissolve completely. Autoclave for 15 minutes at 121°C. Cool to 50°C and aseptically dispense in 50mm sterile petri plates. Medium may be stored at 4°C for 1 month.

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 pipet 1mL into a 10mL buffered water blank. Mix well using a vortex mixer. With a sterile pipette, transfer 1mL of the diluted sample into the next 10mL

buffered water blank. Continue making dilutions, each time using a fresh pipette to avoid carry-over of sample. Prepare duplicate filters for each concentration or volume filtered.

d) Filter, starting with the greatest dilution (lowest bacterial concentration). Rinse with 10-30mL of sterile buffered water. Turn off vacuum.

e) Aseptically remove the membrane filter from the filter base and place grid side up on the agar plates. Reset if air bubbles are trapped under the filter. Incubate R2A Agar plates in an inverted position for 120 hours at 28°C.

f) Using the stereobinocular microscope and cool white fluorescent lamp, count all colonies appearing after 120 hours incubation. Start examination with the highest dilution plate. Colonies may be difficult to distinguish from the white background because they are clear or white, but light will reflect from the colony surface. The numbers of colonies on higher concentration plates should increase by the reciprocal of the dilution interval (logarithmic in the case of decimal dilutions).

If there is no apparent relationship between numbers of colonies and dilution factors, suspect contamination of materials used for filtration and abandon the sample. For subsequent bacterial taxonomy colonies may be purified on brain heart infusion agar (BHIA).

g) Count colonies on membrane filters at 10 to 15 x magnification. Preferably place petri dish on microscope stage slanted at a 45° angle and adjust the light source vertical to the colonies. Optimal colony density per filter is 20 to 200. If colonies are small and there is no crowding, a higher limit is acceptable. Count all colonies on the membrane when there are 1 to 2, or fewer, colonies per square. For 3 to 10 colonies per square count 5 squares and obtain average count per square. Multiply average count per square by 100 times the reciprocal of the dilution to give colonies per millilitre. If there are more than 20 colonies per square, record count as >2000 times the reciprocal of the dilution. Record counts only when there are discrete separate colonies without spreaders.

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.

Data Analysis

a) Calculate the bacterial density using the following formula:
*CFU/mL=(Mean number of bacteria counted) x (Reciprocal of dilution used)
*Colony forming units

b) Plates with no colonies are reported as less than the calculated value of the single largest volume filtered.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, Section 9215 D.
b) Dutka, B., 1981. Membrane Filtration: Applications, Techniques and Problems. Bernard Dutka (Ed.) Marcel Dekker, Inc. New York.

Revision History

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

Presence-Absence (P-A) Coliform 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 Esculin Azide agar	Black halos surround colonial growth.
	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.

d) 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

Multiple-Tube Technique (MPN) for Total Coliform Bacteria in Fresh Water, Wastewater and Marine Water

Parameter

Coliform, total

Analytical Method and EMS Code

Coliform Total, Confirmed MPN: 0451 X015
Coliform Total, Completed MPN: 0451 2495

Scope

This method describes the statistical estimation of total coliforms density in fresh water, wastewater, and marine water. Multiple-tube fermentation technique is the examination of replicate tubes and dilutions reported in terms of Most Probable Number (MPN) of organisms present. This number, based on certain probability formulas, is an estimate of the mean density of bacteria in the sample. MPN method for coliform 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 48 hours incubation at 35°C. Total coliforms are chosen as indicators of water quality because they will be present and recoverable in septic discharges for longer periods of time than are pathogenic organisms.

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 of 0.01%w/v) to neutralize the bactericidal effect of chlorine. Samples should be kept at 4°C until testing. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum volume required for analysis is 75mL.

Detection Limit

2 MPN/100 ml

Interferences

Residual chlorine in chlorinated water must be neutralized with sodium thiosulfate at time of sampling. Mean recoveries of ATCC cultures of E. coli and Klebsiella pneumoniae in lauryl tryptose broth are 138% and 92% respectively.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 35 ± 0.5°C temperature.
b) Sterile serological disposable pipettes, 1mL and 10mL.
c) Lauryl tryptose broth.
d) Brilliant green lactose bile 2% broth
e) 18mm test tubes with inverted Durham tubes.
f) 20mm test tubes with inverted Durham tubes.
g) Autoclave for steam sterilization of glassware and media.
h) Bunsen burner.
i) Sterile petri plates, 100mm
j) Platinum inoculation loops, 3mm diameter.
k) Gram staining reagents (available commercially)
l) Microscope slides and microscope with oil immersion lens.
m) Buffered water dilution blanks.

Reagents

Note: Whenever possible, use commercial dehydrated media.

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 1.25 mL 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) 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 dehydrated 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 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 71.2g in 1L DI and warm slightly to dissolve completely. Dispense 10mL of 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) BRILLIANT GREEN LACTOSE BILE 2% BROTH:

Peptone	10.0 g
Lactose	10.0 g
Oxgall	20.0 g
Brilliant green	0.0133 g

Add all ingredients to 1L DI water, mix thoroughly, and heat to dissolve. pH should be 7.2 ± 0.2 after sterilization. Before sterilization, dispense 10mL aliquots into 18mm fermentation tubes with an inverted vial in each tube. Close tubes with metal or heat-resistant plastic caps, 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.

g) m ENDO AGAR LES

Bacto yeast extract	1.2 g
Casitone	3.7 g
Thiopeptone	3.7 g
Tryptose	7.5 g
Lactose	9.4 g
Potassium phosphate, dibasic	3.3 g
Potassium phosphate, monobasic	1.0 g
Sodium chloride	3.7 g
Sodium desoxycholate	0.1 g
Sodium lauryl sulfate	0.005 g
Sodium sulfite	1.6 g
Bacto basic fuchsin	0.8 g
Bacto agar	15.0 g

Suspend all ingredients in 1L DI to which has been added 20mL 95% undenatured ethanol. Boil to dissolve completely. Cool to 50°C and dispense into sterile petri plates. Do not autoclave.

h) NUTRIENT AGAR

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g

Suspend all ingredients in 1 L DI water, mix thoroughly, and heat to dissolve. pH should be 6.8 ± 0.2 after sterilization. Before sterilization, dispense in screw capped tubes. After sterilization, immediately place tubes in an inclined position so that the agar will solidify with a sloped surface. Tighten screw caps after cooling and store in a protected, cool storage area.

i) GRAM-STAIN REAGENTS:

1) Ammonium oxalate-crystal violet (Hucker's):

Dissolve 2g crystal violet (90% dye content) in 20mL 95% ethyl alcohol; dissolve 0.8g $(NH_4)_2C_2O_4 \cdot H_2O$ in 80 mL distilled water; mix the two solutions and age for 24 hours before use; filter through paper into a staining bottle.

2) Lugol's solution, Gram's modification:

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

3) Counterstain:

Dissolve 2.5g safranine dye in 100mL 95% ethyl alcohol. Add 10mL to 100mL distilled water.

4) Acetone alcohol:

Mix equal volumes of ethyl alcohol (95%) with acetone.

Procedure

PRESUMPTIVE TEST FOR TOTAL 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 water to be tested. For potable water use five 10mL portions or ten 10mL portions; for nonpotable water use five tubes per dilution (of 10, 1, 0.1mL etc.).

b) 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. Shake sample and dilutions vigorously about 25 times. Inoculate each tube of the set of five with replicate sample volumes in increasing decimal dilutions. Use 10mL buffered water blanks to make decimal dilutions of sample for inoculation. Mix test portions in the medium by gentle agitation.

c) Incubate inoculated tubes at 35 ± 0.5 °C. After 24 ± 2 hours shake each tube gently and examine for gas bubbles and/or acidic growth (distinctive yellow colour). If no gas or acid has formed, re-incubate and re-examine at the end of 48 ± 3 hours. A positive presumptive reaction is indicated by the presence of gas bubbles or acid production. Record the number of positive tubes for each dilution. Submit all presumptive positive tubes showing any amount of gas or acidic growth to the confirmed phase.

CONFIRMED PHASE FOR TOTAL COLIFORMS

a) Gently swirl each presumptive positive tube of lauryl tryptose broth and transfer a loopful of each positive culture to tubes of brilliant green lactose bile 2% broth using a sterile inoculating loop or transfer stick. Gently swirl tubes to ensure mixing of inoculum with medium. If additional primary tubes show acidic growth at the end of a 48 hours incubation period, submit these to the confirmed phase.

b) Incubate the inoculated brilliant green lactose bile broth tubes for 48 ± 3 hours at 35 ± 0.5°C. Formation of gas in any amount in the inverted vial at any time within 48 ± 3 hours constitutes a positive confirmed phase. Calculate the confirmed MPN value from the number of positive brilliant green lactose bile tubes.

Alternative procedure: Use this alternative only for polluted water or wastewater known to produce positive results consistently. If all presumptive tubes are positive in two or more consecutive dilutions within 24 hours, submit to the confirmed phase only the tubes of the highest dilution (smallest sample inoculum) in which all tubes are positive and any positive tubes in still higher dilutions. Submit to the confirmed phase all tubes in which gas or acidic growth is produced after 24 hours.

COMPLETED TEST

a) To establish definitively the presence of coliform bacteria and to provide quality control data, use the completed test on all positive confirmed tubes. Double confirmation into brilliant green lactose bile broth for total coliforms may be used. Consider positive EC broth elevated temperature (44.5°C) results as a positive completed test response. Parallel positive brilliant green lactose bile broth cultures with negative EC broth cultures indicate the presence of nonfecal coliforms and must be submitted to the completed test procedure to obtain an MPN test value.

b) From each tube of brilliant green lactose bile broth showing gas, streak one LES-Endo agar plate. Streak plates in a manner to insure presence of some discrete colonies. Incubate plates (inverted) at 35 ± 0.5° C for 24 ±2 hours.

c) From each of the LES-Endo agar plates transfer a typical coliform colony (pink to dark red with a green metallic surface sheen) to a lauryl tryptose broth fermentation tube and a nutrient agar slant.

e) Incubate the slants and secondary lauryl tryptose broth tubes at 35 ±0.5°C. Examine tubes for gas formation at 48 ± 3 hours.

f) Make gram-stained preparations from the agar slants corresponding to the secondary lauryl tryptose broth tubes that show gas formation. (This procedure is usually omitted except with legal samples or if results are doubtful). The formation of gas in the secondary lauryl tryptose broth tube and the demonstration of Gram-negative, non-spore-forming, rod-shaped bacteria in the agar culture constitutes a positive Completed Test.

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 in 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

Multiple-Tube Fermentation Technique (MPN) for Fecal Coliform Bacteria in Fresh Water, Wastewater and Marine Water

Parameter

Coliform, fecal

Analytical Method and EMS Codes

Coliform Fecal Confirmed**: 0450 X015**

Scope

This method describes the probability estimation of the numbers of fecal coliforms from fresh water, wastewater, and marine water. The multiple tube fermentation technique is the examination of replicate tubes and dilutions reported in terms of Most Probable Number (MPN) of organisms present. This number, based on certain probability formulas, is an estimate of the mean density of bacteria in the sample. 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 which are 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. Chlorinated water samples should be treated with sodium thiosulfate (final concentration 0.01% w/v) to neutralize the bactericidal effect of chlorine. Samples should be kept at 4°C until testing. Analysis must begin within 48 hours of sample collection for results to be valid. Minimum volume required for analysis is 75mL.

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 reference materials for fecal coliforms. Mean recoveries of ATCC cultures of E. coli and Klebsiella pneumoniae in lauryl tryptose broth are 138% and 92% respectively.

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.

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) 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 steel closures on tubes and sterilize in autoclave for 15 minutes at 121°C using the liquid cycle. Do not fully open the autoclave door until chamber temperature has dropped below 75°C to avoid trapping air in the inverted vials.

d) LAURYL TRYPTOSE BROTH - DOUBLE STRENGTH

See formula listing above and use twice the weight of each chemical except water.
Suspend 71.2g 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
Monopotassium dihydrogen phosphate, KH_2PO_4	1.5 g
Sodium chloride	5.0 g

Suspend 37g 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 water to be tested. For potable water use five 10mL portions or ten 10mL portions; for nonpotable water use five tubes per dilution (of 10, 1, 0.1mL etc.)

b) 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. Shake sample and dilutions vigorously about 25 times. Inoculate each tube of the set of five with replicate sample volumes in increasing decimal dilutions. Mix dilutions and inoculations by gentle agitation.

c) 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 is 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, 600/8-7-017, 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

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

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

Multiple-Tube Technique (MPN) for Fecal Coliform Bacteria in Bivalve Molluscan Shellfish

Parameter

Coliform, fecal

Analytical Method and EMS Code

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

Scope

This method describes the detection of fecal coliform bacteria in bivalve molluscan shellfish. Diluted samples of blended shellfish are analyzed using the Most Probable Number method to estimate bacterial numbers. The MPN method is applied to health-significant bacteria such as coliforms, fecal 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 fecal coliform levels, as determined by the MPN 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 MPN 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.

Principle

The MPN method is a multiple-tube fermentation technique which estimates the fecal coliform densities in a sample by the pattern of growth and gas formation in the tubes inoculated with various dilutions. Dependent upon the dilution ratio and number of tubes per dilution, the MPN can be calculated based on probability formulas.

Fecal coliforms belong to the larger group of total coliforms, and all are members of the Family Enterobacteriaeceae. Fecal coliforms are Gram-negative, oxidase negative rods which ferment lactose at 44.5°C. Fecal coliforms are selected 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. A fecal coliform test estimates E.coli densities, but the proportion of the other fecal 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 water the incidence of thermotolerant Klebsiella is markedly increased.

Sample Handling

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 sea water that may be found in the sampling area. Shellstock samples should be placed in a 7 - 10 mil thick plastic bag and kept below 10°C, but not frozen. All samples should be analyzed within 24 hours of collection.

Range <20 - 160,000/100gm MPN

Detection Limit A negative result for all inoculated test tubes is <20/100gm.

Interferences None

Apparatus and Materials

a) 25mL wide mouth serological pipettes.
b) 10mL serological pipettes.
c) 1mL serological pipettes.
d) Sterile shucking knives.
e) Sterile brushes.
f) Sterile blender jars.
g) Blender with timer.
h) Incubator capable of maintaining 35 ± 0.2°C.
i) Waterbath capable of maintaining 44.5 ± 0.2°C.
j) 20mm test tubes with inverted Durham tubes.
k) 16mm test tubes with inverted Durham tubes.
l) Autoclave for steam sterilization.
m) Sterile buffered dilution water.

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 SULPHATE SOLUTION

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

c) BUFFERED DILUTION WATER (DILUENT)

Add 1.25 mL stock phosphate buffer solution and 5.0mL magnesium sulphate 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	20.00 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 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 500mL. Dissolve and dispense 10mL of medium per tube, both single and double strength. Tubes should be of

sufficient capacity to contain 1 mL inoculum + 10ml single strength broth or 10mL inoculum + 10mL double strength broth. The pH of the medium should be approximately 6.8 after autoclave sterilization, 15 psi/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 5mL medium into each tube. The pH of the medium should be 6.9 after autoclave sterilization, 15 psi/15 minutes.

Procedures

a) Scrub hands and/or 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 hands and/or gloves with 70% alcohol and then rinse with potable water.

d) Shuck 10 shellstock, 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 at least 5 replicate tubes per sample volume and 3 dilutions.

j) The 1:10 dilution should be shaken 30 times prior to the inoculation of a multiple tube series of 5:5:5. If larger coliform numbers are expected, further decimal dilutions should be made.

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) Incubate tubes at 35°C for 24 hours. Gas production showing in the Durham tubes is regarded as a positive result. Gently tap the cap of any test tubes showing turbidity but no gas production. Re-incubate negative tubes a further 24 hours.

m) 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 waterbath at 44.5°C and ensure the water level is higher than the level of the medium in the test tubes. Incubate the tubes for 24 hours.

n) Positive results are EC tubes showing turbidity and gas production.

o) Remove 48 hour LTB tubes from incubator and check for turbidity and gas production. Repeat procedure 13 as required.

p) All positive EC tubes are used to calculate the MPN value.

Data Analysis

Use the MPN Index to determine fecal coliform levels. Refer to section 4.6 of the Microbiological Quality Assurance/Quality Control section of this manual.

Quality Control

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.

References

a) Standard Methods for the Examination of Water and Wastewater, APHA, AWA, WPCF, 17th edition, 1989, section 9221.
b) *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.
c) Greenberg, A.E. and Hunt, D.A. (eds). Laboratory Procedures for the Examination of Seawater and Shellfish. 5th edition. APHA, 1985.

Revision History

November 14, 1994: Publication in the 1994 Lab Manual

November 14, 2002: SEAM Codes replaced by EMS Codes

Detection of Total Coliforms by Membrane Filtration Fresh Water, Wastewater and Marine Water

Parameter

Coliform, total

Analytical Method and EMS Code

Membrane filter: **0451 X022**

Scope

This method describes the selective isolation of total 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 medium (m Endo) for 18 - 24 hours incubation at 35°C for growth of total coliform colonies.

Principle

Coliforms are Gram negative, non-spore forming, oxidase negative, fermentative rods belonging to the Family Enterobacteriaceae. This group is widely distributed in nature, and many can live as saprophytes in addition to being associated with the intestinal tract of most animals. Coliforms are divided into two groups for convenience. The larger group, which includes all members of the Family Enterobacteriaceae, are called total coliforms, and are defined by their ability to ferment lactose within 24 - 48 hours incubation at 35°C. A sub group of total coliforms are called fecal coliforms, and are defined by their ability to ferment lactose at elevated incubation temperatures. Fecal coliforms are primarily Escherichia coli, although Klebsiella pneumoniae can occasionally grow at 44.5°C. Total coliforms are chosen as indicators of water quality because, although they are not usually pathogens themselves, they will be present and recoverable in septic discharges for longer periods of time than are pathogenic organisms.

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

Precision

There are no standard reference materials for total coliforms. Mean recoveries of ATCC cultures of E. coli and Klebsiella pneumoniae on m Endo are 84% and 95% respectively.

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) Sterile 100mL or 50mL glass graduated cylinders.
d) Sterile disposable petri dishes, 50mm x 12mm with tight fitting lids.
e) Sterile absorbent pads, 50mm.
f) Sterile disposable petri dishes, 100mm x 15mm.
g) m Endo broth MF.
h) BHI agar.
i) Lauryl tryptose broth in 18mm 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) 250mL glass filtration units (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
100 mL 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.

e) m ENDO BROTH MF, dehydrated (DIFCO)

Formula (grams per litre):

Bacto tryptose	10.00 g
Bacto casitone	5.00 g
Bacto yeast extract	1.50 g
Bacto thiopeptone	5.00 g
Bacto lactose	12.50 g
Sodium desoxycholate	0.10 g
Sodium chloride	5.00 g
Dipotassium hydrogen phosphate	4.375 g
Potassium dihydrogen phosphate	1.375 g
Sodium lauryl sulfate	0.050 g
Sodium sulfite	2.10 g
Bacto basic fuchsin	1.05 g

Suspend 4.8g powdered medium in 100mL DI containing 2mL 95% ethanol and heat, just to boiling point. Remove from heat immediately. DO NOT AUTOCLAVE. Cool to room temperature and store for no longer than 72 hours at 4°C. Dispense 2mL onto sterile, absorbent pads in 50mm petri plates immediately prior to use. Final pH of medium is 7.2 at 25°C.

f) LAURYL TRYPTOSE BROTH (DIFCO)

Formula (grams per litre):

Bacto tryptose	20.00 g
Bacto lactose	5.00 g
Dipotassium hydrogen phosphate	2.75 g
Potassium dihydrogen phosphate	2.75 g
Sodium chloride	5.00 g
Sodium lauryl sulfate	0.10 g

Suspend 35.6g 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.

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
Agar	15.0 g

Suspend 52g in 1L DI 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 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 pad saturated with m Endo broth. 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 Endo media plates for 20 - 24 hours at 35± 0.2°C.

h) Count red colonies with golden-green metallic sheen appearing after 20-24 hours incubation.

i) For confirmation, transfer at least 10 colonies to 10 tubes of lauryl tryptose broth and incubate for 24-48 hours at 35°C and observe gas formation in Durham tubes. Alternately, colonies may be purified on BHIA and identified using API® strips according to directions provided by Analytab Products, Inc.

j) Do not count red colonies without sheen, or black colonies.

Data Analysis

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

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

*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 100 mL 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. 59-65, 1989.

Revision History

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

Membrane Filter Technique (MF) for Fecal Coliform Bacteria in Fresh Water, Wastewater and Marine Water

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

Detection of Escherichia Coli by Membrane Filtration in Fresh and Marine Water

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

Detection of Total Coliforms and E. coli by Colilert®

Parameter
Coliform, total
E. Coli

Analytical Method and EMS Codes
Coliform total by Colilert : **0451 X388**
E. coli by Colilert**: 0147 X388**

Scope
This method describes the selective isolation of total coliforms and E.coli from drinking water. Colilert® medium is added to water samples and incubated for 18 - 24 hours at 35°C for growth of total coliforms and E. coli. Colilert® may be used as a presence/absence (P-A) or semi-quantitative 5 tube multiple tube fermentation (MPN) test. The advantages of a rapid, qualitative test for drinking water which can confirm the presence of E. coli outweigh the need for quantitation.

Principle
Coliforms are Gram negative, non-spore forming, oxidase negative, fermentative rods belonging to the family Enterobacteriaceae. This group is widely distributed in nature, and many can live as saprophytes in addition to being associated with the intestinal tract of most animals. Coliforms are divided into two groups for convenience. The larger group, which includes all members of the Family Enterobacteriaceae, are called total coliforms, and are defined by their ability to ferment lactose within 24 - 48 hours incubation at 35°C. Escherichia coli is a fecal coliform, and the presence of E. coli in drinking water is considered to be a strong indication of recent fecal contamination. Colilert® is a system that allows better recovery of stressed E. coli than currently acceptable media, and differentiates E. coli from other total coliforms by the addition of the substrate 4-methylumbelliferyl-B-D-glucuronide (MUG). Coliforms are chosen as indicators of water quality because, although they are not usually pathogens themselves, they will be present and recoverable in septic discharges for longer periods of time than are pathogenic organisms. The presence of E. coli in drinking water is a significant finding.

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 between 4 -10°C until analysis. Variations in temperature are to be avoided. Analysis must begin within 24 hours of sample collection for results to be valid. Minimum volume required for analysis is 125mL.

Range
Positive or Negative for P-A; <2 - >16 for 5 tube MPN per 100mL.

Detection Limit
1 organism/100mL for P-A; <2 for 5 tube MPN per 100mL.

Interferences

Do not use for marine water. MUG negative E. coli will be scored as a total coliform. Chlorinated water must be neutralized with sodium thiosulfate at time of sampling.

Precision

There are no standard reference materials for total coliforms. Mean recoveries of ATCC cultures of E. coli are >100% at 2.5 and 30 CFU/100mL.

Apparatus and Materials

a) Incubator that is capable of maintaining a stable 35 ± 0.5°C temperature.
b) Sterile disposable serological pipettes, 10mL.
c) Sterile 100mL or 50mL glass graduated cylinders.
d) Colilert® medium (available commercially from Nortech Control Equipment).
e) Long wave UV lamp (365 nm).
f) Colour and fluorescence comparator (available commercially from Nortech Control Equipment.)
g) Sterile 20mm test tubes with stainless steel closures.
h) Autoclave for steam sterilization of glassware and bacterial cultures.
i) Bunsen burner
j) API 20E® reagent strips.

Reagents

a) Colilert® is commercially supplied as a preweighed powder. Do not autoclave. Store away from direct light at 4 - 30°C for up to 15 months from date of manufacture.

Procedure

PRECAUTIONS

a) Avoid contaminating the reagent or the inside of the tubes or caps.

b) Thoroughly mix all samples immediately prior to inoculation.

c) Do not autoclave Colilert®. This process would destroy the reagent, which is heat labile.

d) Colilert® is a direct water test only. Do not use for pre-enriched culture procedures. Do not use for confirmation of colonies grown on other selective or non-selective media. Do not concentrate water sample by filtration.

e) Do not dilute the sample in buffered water for addition to Colilert®. Colilert® is already buffered and additional buffer compounds can adversely affect the growth of the target microbes and the test performance.

f) High calcium salt concentrations in certain waters may cause a slight precipitate. This should not affect test results.

g) If a transient blue colour appears upon addition of the water sample to the Colilert® reagent, this is an indication that the water contains some 40-50 times the amount of free chlorine normally found in drinking water. The sample should be considered invalid and testing discontinued.

h) Additional confirmation is normally unnecessary for positive Colilert® tubes. However, growth from positive tubes may be streaked for isolated colonies onto agar plates for bacterial identification using API® 20E strips according to the manufacturer's directions.

SEMI-QUANTITATIVE TEST PROCEDURE

a) Aseptically fill each of five Colilert® tubes with 10mL of a well-mixed water sample.

b) Cap the tubes tightly.

c) Mix vigorously to dissolve the reagent by repeated inversion. Some particles may remain undissolved. Dissolution will continue during incubation. A few reagent salt particles may remain undissolved throughout the test; this will not affect the test performance.

d) Incubate inoculated reagent tubes at 35 ± 0.5°C for 24 hours.

e) Read tubes at 24 hours. Compare each tube against the colour comparator. Colour should be uniform throughout the tube. If not, mix by inversion before reading. If yellow colour is seen, the test is positive for total coliforms. Check for fluorescence by darkening the room, and shining a long wave UV light source 3 - 5 inches from the tubes. If fluorescence of tube(s) is greater than or equal to the fluorescence of the comparator, the presence of E. coli is specifically confirmed.

f) If sample is yellow after 24 hours of incubation, but lighter than the positive comparator tube, it may be incubated up to an additional 4 hours. If the sample is coliform positive, the colour will intensify. If it does not intensify, consider the sample negative.

Some water samples containing humic material may have an innate colour. If a water sample has background colour, compare inoculated Colilert® tubes to a control blank of the same water sample.

g) To find the concentration of total coliforms or E. coli per 100mL, compare the number of positive tubes per sample to the Most Probable Number probability chart below.

MPN Index and 95% Confidence Limits for
Various Combinations of Positive and Negative
Results When Five-10 mL Portions are Used.

# of Tubes Positive Out of 5	MPN Index per 100 mL	95% Confidence limits	
		Upper	Lower
0	<2	0	6.0
1	2	0.1	12.6
2	5	0.5	19.2
3	9	1.6	29.4
4	16	3.3	52.9
5	>16	8.0	Infinite

PRESENCE/ABSENCE TEST (P-A) TEST PROCEDURE

a) Aseptically open a tube of Colilert® reagent and add the contents to a 100 mL water sample in a sterile, transparent, non-fluorescent borosilicate glass container or equivalent. Aseptically cap and seal the vessel.

b) Shake vigorously by repeated inversion to aid dissolution of the reagent. Some particles may remain undissolved. Dissolution will continue during the incubation.

c) Incubate reagent and sample mixture at 35°C ± 0.5°C for 24 hrs.

d) Read the reaction at 24 hours. Compare each reaction vessel against the colour comparator. If no yellow is observed, the test is negative for total coliforms and E. coli. If the sample has a yellow colour greater than or equal to the comparator, the presence of total coliforms is confirmed. If a yellow colour is observed, check each vessel for fluorescence by placing it 3 - 5 inches from a U.V. lamp source in a darkened room. Positive fluorescence greater or equal in intensity to the comparator vessel specifically confirms the presence of E. coli.

e) If sample is yellow after 24 hours of incubation, but lighter than the positive comparator tube, it may be incubated up to an additional 4 hours. If the sample is coliform positive, the colour will intensify. If it does not intensify, consider the sample negative. Some water samples containing humic material may have an innate colour. If a water sample has background colour, compare inoculated Colilert® tubes to a control blank of the same water sample.

Data Analysis

a) MPN: Report total coliform as in MPN table for number of tubes exhibiting yellow colour after 24 hour incubation.

b) MPN: Report E. coli as in MPN table for number of tubes exhibiting fluorescence after 24 hour incubation.

c) P-A: Report positive Total Coliform for samples exhibiting a yellow colour after 24 hour incubation. Report negative total coliform for samples exhibiting no colour after 24 hour incubation.

d) P-A: Report positive E. coli for samples exhibiting fluorescence after 24 hour incubation. Report negative E. coli for samples exhibiting no fluorescence after 24 hour incubation.

Quality Control

a) Grow the following ATCC (American Type Culture Collection) cultures overnight in 10 mL aliquots of BHI broth: ATCC 25922 Escherichia coli, ATCC 13883 Klebsiella Pneumoniae, and ATCC 27853 Pseudomonas aeruginosa.

b) Aseptically empty contents of a tube of Colilert® into a sterile vessel containing 100 mL sterile, deionized water. Mix thoroughly to aid dissolution.

c) Aseptically aliquot 1/3 of the sterile water/reagent mixture into each of three sterile transparent, non-fluorescent borosilicate glass vessels.

d) Aseptically transfer a loop of each ATCC culture to one of the vessels of Colilert® water. Incubate at 35 ± 0.5°C for 24 hours.

e) Results should be observed within 24 hours as follows:

E. coli	=	Yellow and fluorescent
K. pneumoniae	=	Yellow, no fluorescence.
P. aeruginosa	=	No colour, no fluorescence

References

a) Edberg, S.C., Ludwig, F. and Smith, D. B. The Colilert® System for Total Coliforms and Escherichia coli. AWWA Research Foundation, American Water Works Association, Denver, Colorado, 1991.

b) Booth, R.L. Drinking Water Supply Alternate Test Procedure (ATP) -- Action Memorandum. Environmental Monitoring and Support Laboratory, Cincinnati,1988.

c) Monograph, "Colilert® PA Presence/Absence." Environetics, Inc. Branford, CT, 1992.

d) Monograph, "Colilert® MPN Pre-Dispensed MPN." Environetics, Inc. Branford, CT, 2992.

Revision History

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

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

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.
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Detection of Fecal Streptococci by Most Probable Number (MPN) Solids, Soils and Sludge

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.

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

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 Membrane Filter Technique (MF) for Fresh Water, Wastewater and Marine Water

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.

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

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.
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Multiple Tube Technique (MPN) for Salmonella in Solids

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

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Detection of Pseudomonas Aeruginosa by Membrane Filtration (MF) From Fresh Water and Waste Water

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 and 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.

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