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Chapter 10 - MICROBIOLOGICAL ANALYSES

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The discharge of wastes from municipal sewers is one of the most important water quality issues world-wide. It is of particular significance to sources of drinking-water. Municipal sewage contains human faeces and water contaminated with these effluents may contain pathogenic (disease-causing) organisms and, consequently, may be hazardous to human health if used as drinking-water or in food preparation. Faecal contamination of water is routinely detected by microbiological analysis.

It is impractical to attempt the routine isolation of pathogens because they are present in relatively small numbers compared with other types of micro-organism. Moreover, there are many types of pathogen and each requires a unique microbiological isolation technique. The approach that has been adopted is to analyse for indicator organisms that inhabit the gut in large numbers and are excreted in human faeces. The presence of these indicator organisms in water is evidence of faecal contamination and, therefore, of a risk that pathogens are present. If indicator organisms are present in large numbers, the contamination is considered to be recent and/or severe.

Bacteria in water are, in general, not present individually, but as clumps or in association with particulate matter. When enumerating bacteria in water it is not the number of individual bacteria present which are counted, but the number of clumps of bacteria or the particles and their associated bacteria. Each clump or particle may have many bacteria associated with it.

10.1 Characteristics of indicator organisms

Total coliforms

The term “total coliforms” refers to a large group of Gram-negative, rod-shaped bacteria that share several characteristics. The group includes thermotolerant coliforms and bacteria of faecal origin, as well as some bacteria that may be isolated from environmental sources. Thus the presence of total coliforms may or may not indicate faecal contamination. In extreme cases, a high count for the total coliform group may be associated with a low, or even zero, count for thermotolerant coliforms. Such a result would not necessarily indicate the presence of faecal contamination. It might be caused by entry of soil or organic matter into the water or by conditions suitable for the growth of other types of coliform. In the laboratory total coliforms are grown in or on a medium containing lactose, at a temperature of 35 or 37 °C. They are provisionally identified by the production of acid and gas from the fermentation of lactose.

Thermotolerant (faecal) coliforms

The term “faecal coliform” has been used in water microbiology to denote coliform organisms which grow at 44 or 44.5 C and ferment lactose to produce acid and gas. In practice, some organisms with these characteristics may not be of faecal origin and the term “thermotolerant coliform” is, therefore, more correct and is becoming more commonly used. Nevertheless, the presence of thermotolerant coliforms nearly always indicates faecal contamination. Usually, more than 95 per cent of thermotolerant coliforms isolated from water are the gut organism *Escherichia coli*, the presence of which is definitive proof of faecal contamination. As a result, it is often unnecessary to undertake further testing to confirm the specific presence of *E. coli*.

In the laboratory thermotolerant coliforms are grown on media containing lactose, at a temperature of 44 or 44.5 °C. They are provisionally identified by the production of acid and gas from the fermentation of lactose.

Nutrient-rich environments may encourage the growth or persistence of some species of thermotolerant coliform other than *E. coli*. This possibility should be considered when, for example, an unusually high result is obtained from water that was thought to be relatively clean. In such a case, the advice of a microbiology laboratory should be sought for the determination of the more specific indicator, *E. coli*.

Faecal streptococci

The presence of faecal streptococci is evidence of faecal contamination. Faecal streptococci tend to persist longer in the environment than thermotolerant or total coliforms and are highly resistant to drying. It is, therefore, possible to isolate faecal streptococci from water that contains few or no thermotolerant coliforms as, for example, when the source of contamination is distant in either time or space from the sampling point. Faecal streptococci grow in or on a medium containing sodium azide, at a temperature of 37-44 °C. They are usually detected by the reduction of a dye (generally a tetrazolium-containing compound) or the hydrolysis of aesculin. Routine methods may give “false positives” and additional confirmatory tests may be required.

Table 10.1 Comparison of methods for analysis of coliform bacteria

Multiple fermentation tube technique	Membrane filter technique
Slower: requires 48 hours for a positive	More rapid: quantitative results in or presumptive positive about 18 hours
More labour-intensive	Less labour-intensive
Requires more culture medium	Requires less culture medium
Requires more glassware	Requires less glassware
More sensitive	Less sensitive
Result obtained indirectly by statistical approximation (low precision)	Results obtained directly by colony count (high precision)
Not readily adaptable for use in the field	Readily adapted for use in the field
Applicable to all types of water	Not applicable to turbid waters
Consumables readily available in most countries	Cost of consumables is high in many countries
May give better recovery of stressed or damaged organisms in some circumstances	

Heterotrophic plate count

The heterotrophic plate count includes all of the micro-organisms that are capable of growing in or on a nutrient-rich solid agar medium. Two incubation temperatures and times are used: 37 °C for 24 hours to encourage the growth of bacteria of mammalian origin, and 22 °C for 72 hours to enumerate bacteria that are derived principally from environmental sources. The main value of colony counts lies in comparing the results of repeated samples from the same source. If levels increase substantially from normal values, there may be cause for concern.

10.2 Selecting a bacteriological analytical technique

Two techniques are commonly used to detect the presence of coliforms in water. The first of these is called the “multiple fermentation tube” or “most probable number” technique. In this method measured portions of a water sample are placed in test-tubes containing a culture medium. The tubes are then incubated for a standard time at a standard temperature. In the second technique, a measured volume of sample is passed through a fine filter that retains bacteria. The filter is then placed on culture medium and incubated. This is called the “membrane filter” technique. Features of the two techniques are compared in Table 10.1.

10.3 Multiple fermentation tube technique

The technique has been used for the analysis of drinking-water for many years with satisfactory results. It is the only procedure that can be used if water samples are very turbid or if semi-solids such as sediments or sludges are to be analysed. The procedure followed is fundamental to bacteriological analyses and the test is used in many countries.

It is customary to report the results of the multiple fermentation tube test for coliforms as a most probable number (MPN) index. This is an index of the number of coliform bacteria that, more probably than any other number, would give the results shown by the test. It is not a count of the actual number of indicator bacteria present in the sample.

Principle

Separate analyses are usually conducted on five portions of each of three serial dilutions of a water sample. The individual portions are used to inoculate tubes of culture medium that are then incubated at a standard temperature for a standard period of time. The presence of coliforms is indicated by turbidity in the culture medium, by a pH change and/or by the presence of gas. The MPN index is determined by comparing the pattern of positive results (the number of tubes showing growth at each dilution) with statistical tables. The tabulated value is reported as MPN per 100 ml of sample.

There are a number of variants to the multiple fermentation tube technique. The most common procedure is to process five aliquots of water from each of three consecutive 10-fold dilutions; for example, five aliquots of the sample itself, five of a 1/10 dilution of the sample and five of a 1/100 dilution. Aliquots may be 1-ml volumes, each added to 10 ml of single-strength culture medium, or 10-ml volumes, each added to 10 ml of double-strength medium. Results are compared with values such as those given in Table 10.5 (see later).

The use of one of the following variants of the technique may help to reduce the cost of analysis:

• A smaller number of tubes is incubated at each dilution, for example three instead of five. A different table must then be used for the MPN determination (see Table 10.6 later). Some precision is lost, but using 9 tubes instead of 15 saves materials, space in the incubator, and the analyst's time.

• For samples of drinking-water, one tube with 50 ml of sample and five tubes with 10 ml of sample are inoculated and incubated. The results are compared with the values such as those given in Table 10.7 (see later) to obtain the MPN.

10.3.1 Culture media and buffered dilution water

Each part of the test requires a different type of medium. For example, when enumerating coliforms, lauryl tryptose (lactose) broth is used in the first (isolation or presumptive) part of the test. In the second (confirmation) part, brilliant green lactose bile (BGLB) broth is used to confirm total coliforms and *E. coli* medium to confirm faecal coliforms. Some of the characteristics of these and other media suitable for use in most probable number analyses are described in Table 10.2. Media can be made from primary ingredients but are also available in the following forms:

• Dehydrated powder, packaged in bulk (200 g or more), to be weighed out when the medium is prepared and dissolved in an appropriate volume of distilled water, dispensed to culture tubes and sterilised before use.

• Dehydrated powder, packaged in pre-weighed amounts suitable for making one batch of medium, to be dissolved in an appropriate volume of distilled water, dispensed to culture tubes and sterilised before use.

• Ampoules of solution, ready to use.

The ampoules of ready-to-use media are the most convenient form but are the most expensive and have the shortest shelf-life. Pre-weighed packages are easy to use and reduce the risk of error in making up a batch of medium. Media are inevitably expensive when packaged in small quantities. However, they are not a major component of the cost of bacteriological analysis, and the extra cost may be negligible when compared with the greater convenience and reduced wastage. Large bottles containing dehydrated media must be tightly resealed after use to prevent spoilage; this is especially important in humid environments.

Media should be stored in a cool, dark, dry place. After a medium has been prepared by dissolving the powder in distilled water, it should be distributed into culture tubes or bottles and sterilised. Batches of media should be tested before use, using a known positive and negative control organism. If the appropriate reactions are not observed, the media and the control organisms should be investigated and the tests repeated. Media should be used immediately but may be stored for several days provided that there is no risk of their becoming contaminated.

A stock solution of buffered dilution water is prepared by dissolving 34.0 g of potassium dihydrogen phosphate, KH_2PO_4, in 500 ml of distilled water. The pH is checked and, if necessary, adjusted to 7.2 by the addition of small quantities of 1 mol l^{-1} NaOH solution. Distilled water is added to bring the final volume to 1 litre. The buffered water is stored in a tightly stoppered bottle in the refrigerator.

To prepare bottles of dilution water, 1.25 ml of stock solution is added to 1 litre of distilled water, mixed well and dispensed into dilution bottles in quantities that will provide, after sterilisation, 9 or 90 ml. The bottles are loosely capped, placed in the autoclave, and sterilised for 20 minutes at 121 °C. After the bottles have been removed from the autoclave, the caps should be tightened and the bottles stored in a clean place until needed.

Table 10.2 Culture media for most probable number (MPN) analyses

Medium	Uses	Incubation temperature	Remarks
Isolation media			
Lactose broth	Total or thermotolerant coliforms	48 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms	Prepare single strength medium by diluting double strength medium with distilled water. Each tube or bottle should contain an inverted fermentation (Durham) tube
MacConkey broth	Total or thermotolerant coliforms	48 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms	
Improved formate lactose glutamate medium	Total or thermotolerant coliforms	48 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms	Available commercially in dehydrated form as Minerals Modified Glutamate Medium
Lauryl tryptose (lactose) broth	Total or thermotolerant coliforms	48 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms	
Confirmatory media			
Brilliant green lactose bile broth	Total or thermotolerant coliforms (gas production)	44.5 ± 0.25 °C for thermotolerant coliforms	
EC medium	Thermotolerant coliforms (indole production)	44.5 ± 0.25 °C for thermotolerant coliforms	Addition of 1 % (m/m) L- or DL-tryptophan may improve performance of the medium
Tryptone water	Thermotolerant coliforms (gas + indole production)	44.5 ± 0.25 °C for thermotolerant coliforms	
Lauryl tryptose mannitol broth with tryptophan	Thermotolerant coliforms (gas + indole production)	44.5 ± 0.25 °C for thermotolerant coliforms	

Source: Adapted from ISO, 1990b

10.3.2 Procedure

The procedure described below is for five tubes at each of three sample dilutions and provides for confirmation of both total and thermotolerant (faecal) coliforms. If fewer tubes (e.g. three of each of three sample dilutions or one 50-ml and five 10-ml portions) are inoculated, the MPN index must be determined from tables specific to the combination of tubes and dilutions used.

Apparatus

√ Incubator(s) or water-baths capable of maintaining a temperature to within ± 0.5 ºC of 35 and 37 ºC and to within ± 0.25 ºC of 44 and 44.5 ºC. The choice of temperature depends on the indicator bacteria and the medium.

√ Autoclave for sterilising glassware and culture media. The size required depends on the volume of work to be undertaken. A capacity of 100-150 litres would be required for a medium-size laboratory undertaking work on a routine basis.

√ Distillation apparatus, with storage capacity for at least 20 litres of distilled water.

√ Laboratory balance, accuracy ± 0.05 g, with weighing scoop. This may be omitted if culture media and potassium dihydrogen phosphate are available in pre-weighed packages of the proper size.

√ Racks for tubes and bottles of prepared culture media and dilution water. These must fit into the autoclave.

√ Pipettes, reusable, glass, 10-ml capacity graduated in 0.1-ml divisions, and 1-ml capacity graduated in 0.01-ml divisions.

√ Test-tubes, 20 × 150 mm for 10 ml of sample + 10 ml of culture medium, with metal slip-on caps.

√ Bottles, with loose-fitting caps, calibrated at 50 and 100 ml, for 50 ml of sample + 50 ml of culture medium.

√ Measuring cylinders, unbreakable plastic or glass, capacity 100, 250, 500 and 1,000 ml.

√ Test-tube racks to hold tubes in incubator and during storage.

√ Thermometer for checking calibration of incubator or water-bath.

√ Refrigerator for storage of prepared culture media.

√ Hot-air steriliser for sterilising pipettes.

√ Bunsen burner or alcohol lamp.

√ Durham tubes, 6 × 30 mm.

√ Pipette cans for sterilising pipettes.

√ Flasks for preparation of culture media.

√ Wash-bottle.

√ Pipette bulbs.

√ Wire loops for inoculating media, and spare wire.

√ Spatula.

√ Container for used pipettes.

√ Brushes for cleaning glassware (several sizes).

√ Fire extinguisher and first-aid kit.

√ Miscellaneous tools.

√ Waste bin.

Consumables
√ Culture media: for example lauryl tryptose broth, brilliant green lactose bile (BGLB) broth, and *E. coli* medium.

√ Disinfectant for cleaning laboratory surfaces and the pipette discard container.

√ Detergent for cleaning glassware and equipment.

√ Phosphate-buffered dilution water.

√ Autoclave tape.

Table 10.3 Typical sample volumes and number of tubes for multiple fermentation tube analysis

Sample type	Sample volume (ml)				
	50	**10**	**1**	**0.1**	**0.01**
Treated drinking-water	1	5			
Partially treated drinking-water		5	5	5	
Recreational water		5	5	5	
Protected-source water		5	5	5	
Surface water			5	5	5

Volumes of 0.1 and 0.01 ml of sample are obtained by addition of 1 ml of a 1/10 and 1/100 dilution, respectively, of the sample to 10 ml of single-strength culture medium.

Procedure

Note: Aseptic technique must be used.

1. Prepare the required number of tubes of culture medium. The volume and strength (single or double) of medium in the tubes will vary depending on the expected bacteriological density in the water and the dilution series planned. For most surface waters, 10 ml volumes of single-strength medium are appropriate.

2. Select and prepare a range of sample dilutions; these will normally be suggested by experience. Recommended dilutions for use when there is no experience with samples from that station are given in Table 10.3. To prepare a 1/10 dilution series, mix the sample bottle

well. Pipette 10 ml of sample into a dilution bottle containing 90 ml of phosphate-buffered dilution water. To prepare a 1/100 dilution, mix the 1/10 dilution bottle well and pipette 10 ml of its contents into a bottle containing 90 ml of dilution water. Subsequent dilutions are made in a similar way. Alternatively, 1 ml of sample may be added to a bottle containing 9 ml of dilution water.

3. Pipette the appropriate volumes of sample and diluted sample into the tubes of medium, as shown in Figure 10.1a.

4. Label the tubes with the sample reference number, the dilution and the volume of sample (or dilution) added to the tube. Shake gently to mix the sample with the medium. Place the rack in an incubator or water-bath for 48 hours at 35 ± 0.5 ºC or 37 ± 0.5 ºC.

5. After 18 or 24 hours, note which tubes show growth. The reactions are listed in Table 10.4. Tubes that show turbidity and gas production, or a colour change indicating the production of acid (if the medium contains a pH indicator), are regarded as positive. Record the number of positive tubes at each dilution, as shown in Figure 10.1b. Return the tubes to the incubator and re-examine after a total of 48 hours of incubation. Continue with the next step of the procedure.

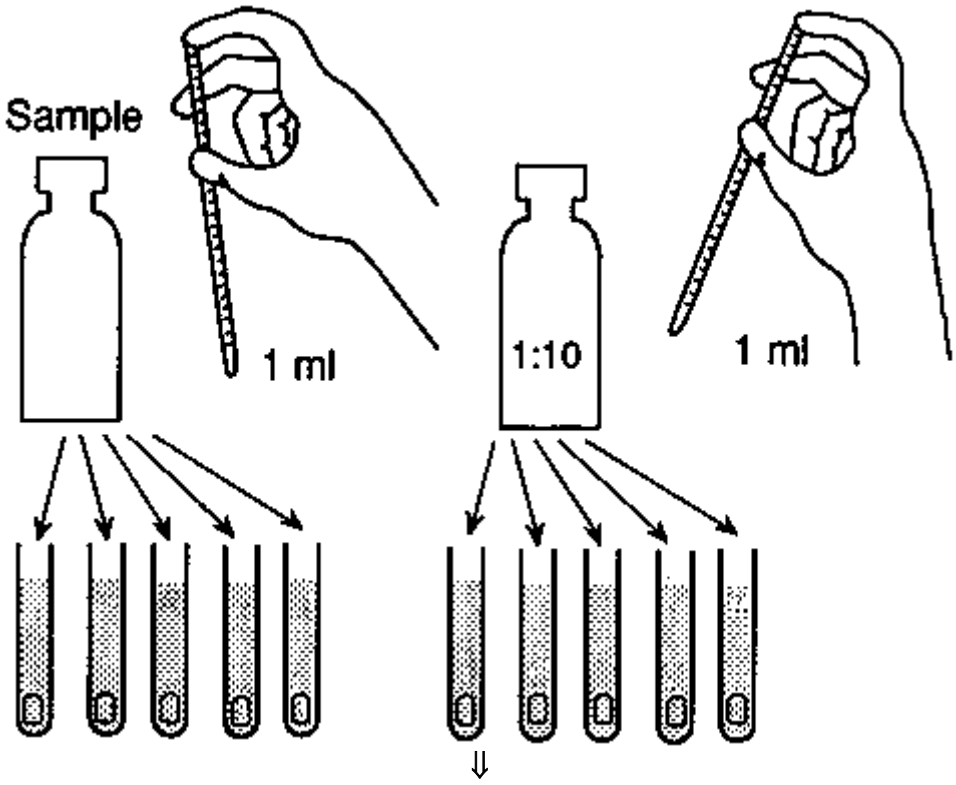
6. Prepare the required number of tubes of confirmation culture medium (BGLB broth for total coliforms and *E. coli* medium for faecal coliforms). Using a sterile wire loop, transfer inocula from positive tubes into the confirmation medium, as shown in Figure 10.1c. Sterilise the loop between successive transfers by heating in a flame until it is red hot. Allow it to cool before use. If confirmation of both total and faecal coliforms is required, a BGLB and an *E. coli* medium tube should be inoculated from each presumptive positive. Label these tubes carefully with the same code used in the presumptive test and incubate them for 48 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms (BGLB broth) or for 24 hours at 44 ± 0.5 °C for faecal coliforms (*E. coli* medium).

7. After the prescribed incubation time, note which tubes show growth with the production of gas, and record the number of positives for each sample dilution as shown in Figure 10.1d.

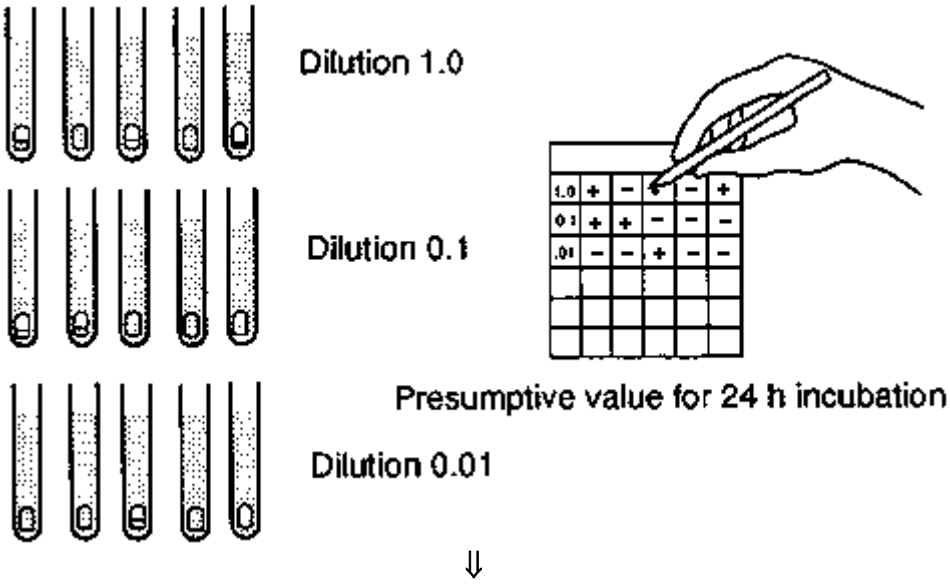
8. Compare the pattern of positive results with a most probable number table such as one of those given in Tables 10.5, 10.6 and 10.7.

Figure 10.1 Steps in the multiple fermentation tube technique

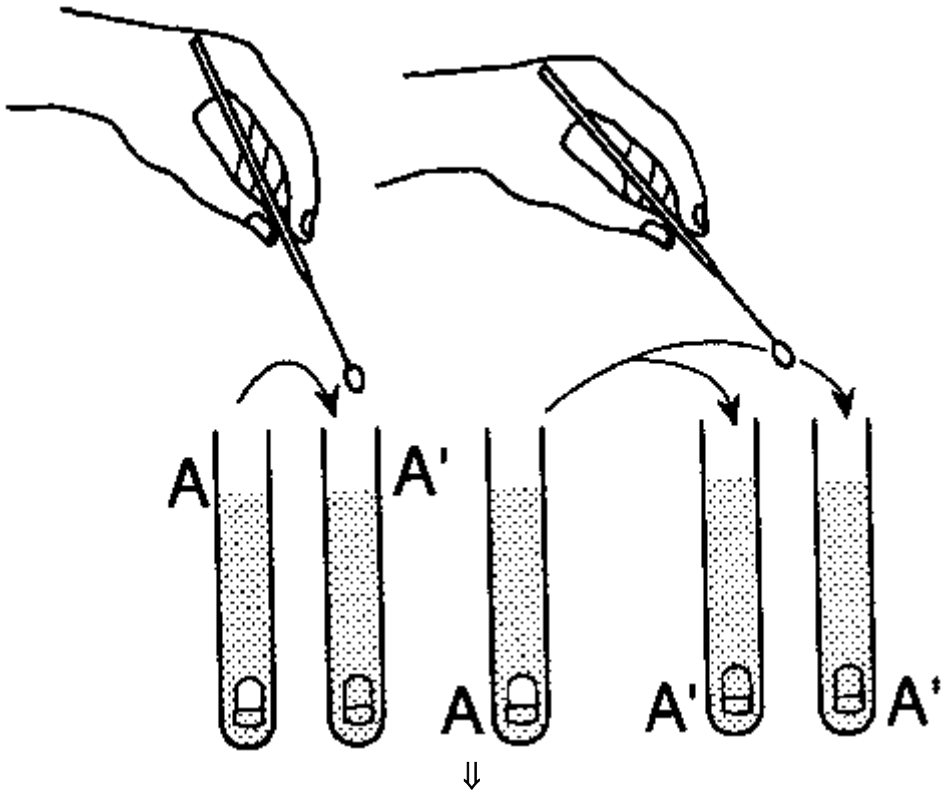
a. Pipette sample into fermentation tubes



b. Calculate presumptive value after 24 hours incubation



c. Inoculate fermentation tube with wire loop for confirmatory test



d. Calculate confirmed test result after complete incubation

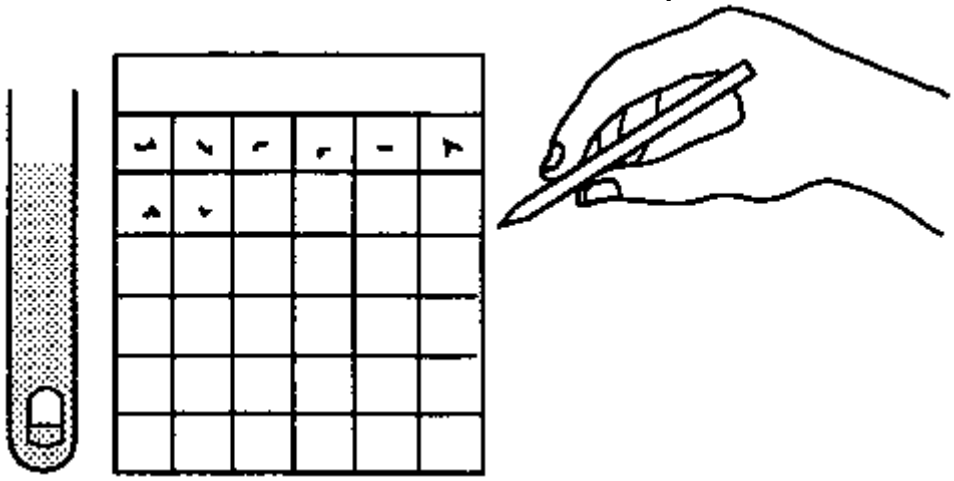


Table 10.4 Reactions following analysis by the MPN method

Medium	Reactions	
	Total coliforms at 35 or 37 °C	**Thermotolerant coliforms at 44 or 44.5 °C**
Isolation media		
Lactose broth	Gas visible in the inverted fermentation (Durham) tube plus turbidity of the medium	Same as total coliforms at 35 or 37 °C
MacConkey broth	Gas visible in the inverted fermentation (Durham) tube plus turbidity of the medium	Same as total coliforms at 35 or 37 °C
Improved formate lactose glutamate medium	Gas visible in the inverted fermentation (Durham) tube plus turbidity of the medium	Same as total coliforms at 35 or 37 °C
Lauryl tryptose (lactose) broth	Gas visible in the inverted fermentation (Durham) tube plus turbidity of the medium	Same as total coliforms at 35 or 37 °C
Confirmatory media		
Brilliant green lactose bile broth	Gas visible in the inverted fermentation (Durham) tube plus turbidity of the medium	Same as total coliforms at 35 or 37 °C
EC medium	Gas visible in the inverted fermentation (Durham) tube plus turbidity of the medium	Same as total coliforms at 35 or 37 °C
Tryptone water		Add KOVACS' reagent to tube; a red colour denotes presence of indole
Lauryl tryptose mannitol broth with tryptophan		Allows detection of gas + indole production in same tube

Source: Adapted from ISO, 1990b

Table 10.5 MPN index and 95 per cent confidence limits for various combinations of positive results when five tubes are used per dilution (10 ml, 1.0 ml, 0.1 ml portions of sample)

Combination of positives	MPN index per 100 ml	95 % confidence limits		Combination of positives	MPN index per 100 ml	95 % confidence limits	
		Upper	Lower			Upper	Lower
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 4-4-0	33 34	15 16	77 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 5-1-2	50 60	20 30	150 180
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	1,300
4-1-0	17	7.0	46	5-5-2	500	200	2,000
4-1-1	21	9.0	55	5-5-3	900	300	2,900
4-1-2	26	12.0	63	5-5-4 5-5-5	1,600 >1,600	600 -	5,300 -

Source: After APHA, 1992

Table 10.6 MPN index for various combinations of positive results when three tubes are used per dilution (10 ml, 1.0 ml and 0.1 ml portions of sample)

Number of tubes giving a positive reaction from			MPN
3 of 10 ml each	3 of 1 ml each	3 of 0.1 ml each	
0	0	0	<3
0	0	1	3
0	1	0	3
1	0	0	4
1	0	1	7
1	1	0	7
1	1	1	11
1	2	0	11
2	0	0	9
2	0	1	14

Source: After WHO, 1985

Table 10.7 MPN index and 95 per cent confidence limits for various combinations of positive results for a set of one 50 ml and five 10 ml portions of sample

Number of tubes giving positive reaction		MPN Index per 100 ml	95 % confidence limits	
1 × 50 ml	5 × 10 ml		Lower	Upper
0	0	<1		
0	1	1	0.5	4
0	2	2	0.5	6
0	3	4	0.5	11
0	4	5	1	13
0	5	7	2	17
1	0	2	0.5	6
1	1	3	0.5	9
1	2	6	1	15
1	3	9	2	21
1	4	16	4	40
1	5	>18		

Source: After Department of Health and Social Security, 1982

10.4 Membrane filter technique

The membrane filter technique can be used to test relatively large numbers of samples and yields results more rapidly than the multiple fermentation tube technique. It was originally designed for use in the laboratory but portable equipment is now available that permits use of the technique in the field. The names and addresses of several manufacturers of portable equipment are given in Annex 1.

Principle

The membrane filter method gives a direct count of total coliforms and faecal coliforms present in a given sample of water. A measured volume of water is filtered, under vacuum, through a cellulose acetate membrane of uniform pore diameter, usually 0.45 μm. Bacteria are retained on the surface of the membrane which is placed on a suitable selective medium in a sterile container and incubated at an appropriate temperature. If coliforms and/or faecal coliforms are present in the water sample, characteristic colonies form that can be counted directly.

The technique is unsuitable for natural waters containing very high levels of suspended material, sludges and sediments, all of which could block the filter before an adequate volume of water has passed through. When small quantities of sample (for example, of sewage effluent or of grossly polluted surface water) are to be tested, it is necessary to dilute a portion of the sample in sterile diluent to ensure that there is sufficient volume to filter across the entire surface of the membrane.

Suggested volumes to be filtered for water from different sources are listed in Table 10.8. If the quality of water is totally unknown, or there is doubt concerning the probable bacterial density, it is advisable to test two or more volumes in order to ensure that the number of colonies on the membrane will be in the optimum range for counting (i.e. 20-80 colonies per membrane). If a suitable volume of sample cannot be filtered through a single membrane, the sample may be filtered through two or more and the numbers of colonies on the membranes added to give the total count for the sample.

Membrane filtration and colony count techniques assume that each bacterium, clump of bacteria, or particle with bacteria attached, will give rise to a single visible colony. Each of these clumps or particles is, therefore, a colony forming unit (cfu) and the results are expressed as colony forming units per unit volume. In the case of thermotolerant coliform bacteria the result should be reported as thermotolerant coliforms [No.] cfu per 100 ml.

Table 10.8 Typical sample volumes for membrane filtration analysis

Sample type	Sample volume (ml)					
	100	**10**	**1**[1]	**0.1**[1,2]	**0.01**[1,2]	**0.001**[1,2]
Treated drinking water	x					
Partially treated drinking water	x	x				
Recreational water			x	x		
Protected source water		x	x			
Surface water			x	x		
Wastewater			x	x	x	
Discharge from sewage treatment plant			x	x	x	
Ponds, rivers, stormwater runoff				x	x	x
Raw sewage				x	x	x

[1] Small volumes should be added to the filtration apparatus together with a minimum of 9 ml of sterile diluent to ensure adequate dispersal across the surface of the filter membrane
[2] 1.0, 0.1, 0.01 and 0.001-ml volumes are filtered after first preparing serial dilutions of the sample.

To filter:

1.0 ml of sample, use 10 ml of 1/10 dilution
0.1 ml of sample, use 10 ml of 1/100 dilution
0.01 ml of sample, use 10 ml of 1/1,000 dilution
0.001 ml of sample, use 10 ml of 1/10,000 dilution

10.4.1 Culture media and buffered dilution water

The general comments on media in the section devoted to the multiple fermentation tube technique (section 10.3.1) are also relevant here. The media that may be used are described in Table 10.9. A stock solution of buffered dilution water is prepared as described in section 10.3.1 and stored in a tightly stoppered bottle in the refrigerator.

To prepare bottles of dilution water, 1.25 ml of stock solution is added to 1 litre of distilled water, mixed well and dispensed into dilution bottles in quantities that will provide, after sterilisation, 9 ± 0.5 ml. The bottles are loosely capped, placed in the autoclave and sterilised for 20 minutes at 121 °C. After the bottles have been removed from the autoclave, the caps should be tightened and the bottles stored in a clean place until they are needed.

A supply of good-quality distilled water may not be available if membrane filter equipment is being used in the field. Some alternatives are:

- Rainwater, which is normally low in dissolved solids and, if filtered through the membrane filter apparatus, should be free of suspended solids and bacteria. In some areas rainwater may be acidic and, therefore, pH should be checked before the water is used for the preparation of culture media.

- De-ionising packs, which are available from commercial suppliers and, if used in accordance with manufacturers' instructions, will supply good-quality water. They may be expensive and are not readily available in some countries.

- Water for car batteries, which is often available at stations where vehicle fuel is sold. However, it may be of lower purity than desired and should probably be filtered through the membrane filter apparatus.

The quality of distilled water should be checked before use. Conductivity should be less than 10 μmhos cm^{-1} and pH should be close to 10.

Table 10.9 Culture media for membrane filtration

Medium	Uses	Incubation temperature	Remarks
Lactose TTC agar with Tergitol 7	Total or thermotolerant coliforms	18-24 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 18-24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms	Adjust pH before sterilisation. Filter TTC supplement to sterilise. Tergitol supplement sterilised by autoclaving. Supplements of Tergitol and TTC to be added aseptically. Prepared plates have max. shelf-life of 10 days. Store in dark.
Lactose agar with Tergitol 7	Total or thermotolerant coliforms	18-24 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 18-24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms	Prepared plates have max. shelf-life of 10 days. Store prepared plates at 4 °C.
Membrane enrichment with Teepol broth	Total or thermotolerant coliforms	18-24 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 18-24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms	Check pH before sterilisation
Membrane lauryl sulphate broth	Total or thermotolerant coliforms	18-24 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 18-24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms	Check pH before sterilisation
Endo medium	Total coliforms only	35-37 °C	Basic fuchsin may be a carcinogen. Also requires ethanol. Do not autoclave. Prepared medium has a shelf-life of 4 days. Store prepared medium at 4 °C in the dark.
LES Endo medium	Total coliforms only	35-37 °C	Basic fuchsin may be a carcinogen. Also requires ethanol. Do not autoclave. Prepared medium has a shelf-life of 2 weeks. Store prepared medium at 4 °C in the dark.
MFC	Thermotolerant coliforms	44 °C	Do not autoclave. Discard unused medium after 96 hours. Rosalic acid stock solution has a maximum shelf-life of 2 weeks. Check pH before sterilisation. Store prepared medium at 2-10 °C.

Source: Adapted from ISO, 1990a

Culture media and buffered dilution water may be prepared in the field, but this requires the transport of all necessary equipment, which may include measuring cylinders, beakers, distilled water, autoclavable bottles, a large pressure-cooker and a gas burner or other source of heat. Pre-weighed portions of the dehydrated medium should be used. The medium should be mixed with water and dispensed into bottles, each of which should contain enough medium for one day's work. These bottles should be sterilised in the pressure-cooker for 20 minutes. Buffered dilution water should be prepared, dispensed into bottles and sterilised.

Note: Whenever a pressure-cooker is used, care must be taken to ensure that the pressure has dropped to atmospheric pressure before the lid is removed.

10.4.2 Procedure

Apparatus

√ Incubator(s) or water-bath(s) capable of maintaining a temperature to within ± 0.5 °C of 35 and 37 °C and to within ± 0.25 °C of 44 and 44.5 °C. Choice of temperature depends on the indicator bacteria and the medium.

√ Membrane filtration apparatus, complete with vacuum source (electrically operated pump, hand-pump or aspirator) and suction flask.

√ Autoclave for sterilising prepared culture media. A pressure-cooker, heated on a hot-plate or over a Bunsen burner, may be substituted in some circumstances.

√ Boiling-pan or bath (if filtration apparatus is to be disinfected in boiling water between uses).

√ Laboratory balance, accurate to ± 0.05 g, and with weighing scoop. This may be omitted if media and potassium dihydrogen phosphate are available in pre-weighed packages of the correct size.

√ Racks for bottles of prepared culture media and dilution water. These must fit into the autoclave or pressure-cooker.

√ Distilling apparatus with storage capacity for at least 5 litres of distilled water.

√ Refrigerator for storage of prepared culture media.

√ Hot-air steriliser for sterilising pipettes and glass or metal Petri dishes.

√ Thermometer for checking calibration of incubator or water-bath.

√ Pipette cans for sterilising pipettes.

√ Boxes for Petri dishes for use in hot-air steriliser.

√ Reusable bottles for culture media.

√ Measuring cylinders, capacity 100 ml and 250 ml.

√ Reusable pipettes, glass, capacity 1 ml and 10 ml.

√ Bottles to contain 9-ml volumes of buffered dilution water.

√ Flasks for preparation of culture media.

√ Wash-bottle.

√ Blunt-edged forceps.

√ Pipette bulbs.

√ Spatula.

√ Container for used pipettes.

√ Brushes for cleaning glassware (several sizes).

√ Fire extinguisher and first-aid kit.

√ Miscellaneous tools.

√ Waste bin.

Filtration apparatus should be disinfected between analyses of consecutive samples and sterilised at intervals. The choice of method will depend on the type of apparatus, where it is being used, the equipment available and cost considerations. Sterilising is generally achieved by autoclaving but any of the following methods is suitable for disinfection between analyses of consecutive samples:
- immersion of components in boiling water for at least 1 minute,

- rinsing in methanol followed by rinsing in distilled water,

- flaming with methanol, or

- exposure to formaldehyde gas generated by burning methanol in the absence of oxygen (generally preferred as a field technique).

Consumables
√ Methanol for disinfecting filtration apparatus using formaldehyde gas (unnecessary in the laboratory, but essential if analyses are done in the field). It is essential to use methanol. Ethanol or methylated spirits cannot be substituted.

√ Membrane filters, 0.45 μm pore size and of diameter appropriate for the filtration apparatus being used and complete with absorbent pads.

√ Disinfectant for cleaning laboratory surfaces and a container for discarded pipettes.

√ Culture media (options are listed in the section on media).

√ Phosphate-buffered dilution water.

√ Petri dishes, glass or aluminium (reusable) or plastic (disposable).

√ Polyethylene bags for wrapping Petri dishes if dry incubator is used.

√ Magnifying lens (as an aid to counting colonies after filters are incubated).

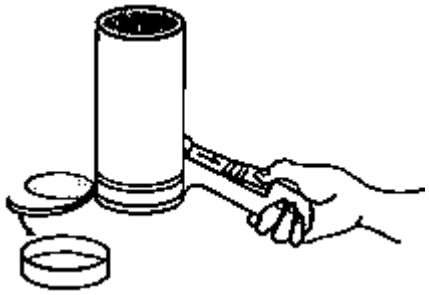
√ Wax pencils for labelling Petri dishes.

√ Autoclave tape.

√ Detergent for cleaning glassware and equipment.

Figure 10.2 Steps in the membrane filter technique

a. Add absorbant pad to Petri dish

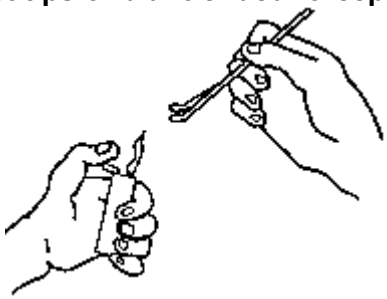


⇓

b. Soak pad in nutrient medium

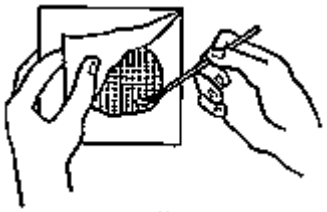
⇓

c. Disinfect tips of blunt-ended forceps and cool



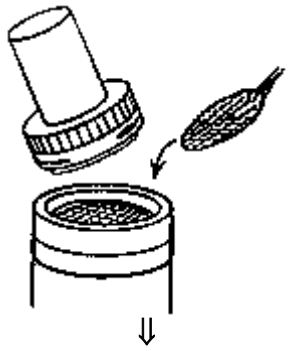
⇓

d. Remove membrane filter from sterile packet

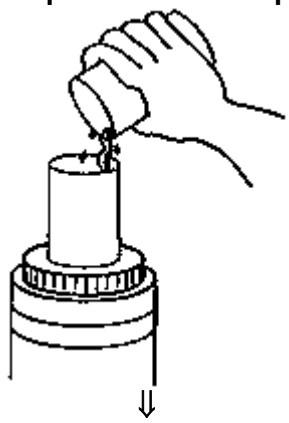


⇓

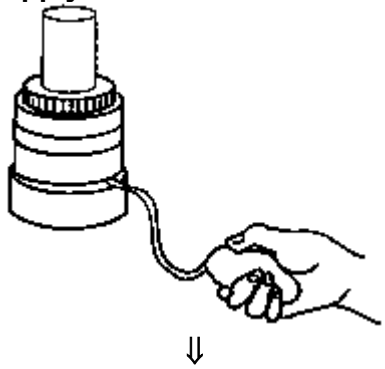
e. Place membrane filter in filtration apparatus



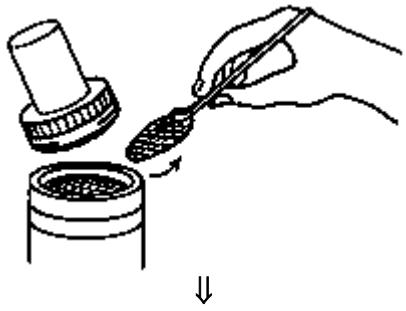
f. Add sample to filtration apparatus



g. Apply vacuum to suction flask



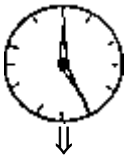
h. Remove filter with sterile forceps



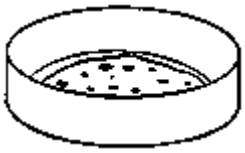
i. Place filter in prepared Petri dish

j. Label Petri dish

k. Leave to resuscitate and then incubate



l. Count colonies after full incubation



Procedure

1. Add absorbent pads to sterile Petri dishes for the number of samples to be processed. Sterile pads may be placed in the Petri dishes with sterile forceps or with an automatic dispenser as shown in Figure 10.2a.

2. Soak the pads with nutrient medium. Nutrient medium may be dispensed with a sterile pipette or by carefully pouring from an ampoule or bottle, as shown in Figure 10.2b. In all cases, a slight excess of medium should be added (e.g. about 2.5 ml). Immediately before processing a sample, drain off most of the excess medium, but always ensure that a slight excess remains to prevent the pad drying during incubation.

Note: Absorbent pads soaked in liquid medium may be replaced by medium solidified by agar. In this case, Petri dishes should be prepared in advance and stored in a refrigerator.

3. Sterilise the tips of the blunt-ended forceps in a flame and allow them to cool (Figure 10.2c).

4. Carefully remove a sterile membrane filter from its package, holding it only by its edge as shown in Figure 10.2d.

5. Place the membrane filter in the filter apparatus as shown in Figure 10.2e, and clamp it in place. If the apparatus has been disinfected by boiling, ensure that it has cooled down before inserting the membrane filter.

6. Mix the sample by inverting its container several times. Pour or pipette the desired volume of sample into the filter funnel (see Figure 10.2f). This volume should normally be chosen in the light of previous experience, but suggested volumes are given in Table 10.8. If the volume to be filtered is less than 10 ml, it should be made up to at least 10 ml with sterile diluent so that the sample will be distributed evenly across the filter during filtration. Alternatively, the sample may be diluted as suggested in footnote 2 to Table 10.8.

7. Apply a vacuum to the suction flask and draw the sample through the filter; disconnect vacuum (Figure 10.2g).

8. Dismantle the filtration apparatus and remove the membrane filter using the sterile forceps, taking care to touch only the edge of the filter (Figure 10.2h).

9. Remove the lid of a previously prepared Petri dish and place the membrane, grid side uppermost, onto the pad (or agar). Lower the membrane, starting at one edge in order to avoid trapping air bubbles. The procedure is shown in Figure 10.2i.

10. Replace the lid of the Petri dish and mark it with the sample number or other identification (see Figure 10.2j). The sample volume should also be recorded. Use a wax pencil or waterproof pen when writing on Petri dishes.

11. If membranes are going to be incubated at 44 or 44.5 °C, the bacteria on them may first require time to acclimatise to the nutrient medium. After processing samples from areas of temperate climate, leave each Petri dish at environmental temperature for 2 hours before placing it in the incubator. Samples from areas of tropical climate may be incubated immediately.

12. Maintain the Petri dish in a humid atmosphere (e.g. in a plastic bag or in a small container with a moist pad in the base) and incubate it either in an incubator or in a weighed canister in a water bath. This ensures that the pad does not dry out during the incubation period.

13. The incubation periods and temperatures required for each culture medium are listed in Table 10.9. Characteristics of total coliform and thermotolerant coliform colonies grown on the various culture media are described in Table 10.10.

14. After incubation, count the colonies. Express the results as number of colonies per 100 ml of sample. Where smaller volumes have been used, results are calculated from the following formula:

No. of colonies per 100 ml = [(No. of colonies)/(volume filtered)] × 100
The colonies counted at this stage are presumed to be coliform bacteria (presumptive results).

Table 10.10 Colony characteristics following analysis by the membrane filtration method

Medium	Colony characteristics	
	Total coliforms at 35 or 37 °C	**Thermotolerant coliforms at 44 or 44.5 °C**
Lactose TTC agar with Tergitol 7	Yellow, orange or brick red coloration with yellow central halo in the medium under the membrane	Same as total coliforms at 35 or 37 °C
Lactose agar with Tergitol 7	Yellow central halo in the medium under the membrane	Same as total coliforms at 35 or 37 °C
Membrane enriched Teepol broth	Yellow colour extending on to the membrane	Same as total coliforms at 35 or 37 °C
Membrane lauryl sulphate broth	Yellow colour extending on to the membrane	Same as total coliforms at 35 or 37 °C
Endo agar or broth	Dark red colour with golden-green metallic sheen	(not applicable)
LES Endo agar	Dark red colour with golden-green metallic sheen	(not applicable)
MFC medium	(not applicable)	Blue colonies

Source: Adapted from ISO, 1990a

10.4.3 Confirmatory tests

For the examination of raw or partly treated waters, presumptive results may be adequate but, in certain other circumstances, it is important to carry out confirmatory tests on pure subcultures.

To confirm the membrane results for total coliforms, each colony (or a representative number of colonies) is subcultured to tubes of lactose peptone water and incubated at 35 or 37 °C for 48 hours. Gas production within this period confirms the presence of total coliforms.

To confirm thermotolerant coliforms and *E. coli* on membranes, whether incubated at 35, 37 or 44 °C, each colony (or a representative number of colonies) is subcultured to a tube of lactose peptone water and a tube of tryptone water. Tubes are incubated at 44 °C for 24 hours. Growth with the production of gas in the lactose peptone water confirms the presence of thermotolerant coliforms. Confirmation of *E. coli* requires the addition of 0.2-0.3 ml of Kovac's reagent to each tryptone water culture. Production of a red colour indicates the synthesis of indole from tryptophan and confirms the presence of *E. coli*.

Note: Use of lauryl tryptose mannitol broth with tryptophan allows both gas production and indole synthesis to be demonstrated in a single tube.

10.5 Quality assurance

Quality assurance is discussed in detail in Chapter 9, which should be read in conjunction with this section. In this section, guidance is given on those aspects of analytical quality control that apply only to microbiological laboratories, particularly the preparation and control of laboratory consumables (media and dilution solutions, membrane filters and pads, plastic- and glassware). The monitoring of laboratory equipment is discussed briefly. Readers who wish to develop more rigorous quality control procedures should refer to the appropriate literature cited in section 10.6, such as the *Standard Methods for the Examination of Water and Wastewater*, which provides detailed guidelines for microbiological quality control.

10.5.1 Laboratory equipment

Chapter 9 deals with monitoring and control of laboratory equipment, maintenance of operational records and use of calibrated instruments for taking measurements. The principles discussed there also apply to equipment used in microbiological laboratories. Particular attention should be paid to incubators, water-baths, refrigerators and freezers, since correct operation is vital to the reliability of test results. It is recommended that temperature measurements are made twice daily: first thing in the morning before equipment is used, and again at the end of the working day.

Incubators, refrigerators and freezers should be cleaned at least once a month. The manufacturers' instructions should include advice on cleaning and may recommend suitable detergents and disinfectants. Water-baths may need more frequent cleaning to control bacterial growth.

10.5.2 Glassware and plasticware

Some detergents and other agents used for the cleaning of laboratory glassware may contain substances that will either inhibit or stimulate the growth of bacteria. Samples of glassware should be checked regularly, for example once a month, if washing procedures and products are always the same. If procedures change, however, or new products are introduced, additional checks should be made. Laboratory plastics may also contain inhibitory residues and each new batch of plasticware should be checked.

Procedure

1. Use a type strain of *E. coli* or a well characterised laboratory isolate. Culture the organism overnight in a nutrient broth at 37 °C, and dilute in quarter-strength Ringer's solution to a concentration of 50-200 colony-forming units per ml.

2. Wash and rinse six glass Petri dishes (glass Petri dishes may also be used to simulate washed glassware) according to usual laboratory practice. Designate these as group A.

3. Wash a further six Petri dishes and rinse 12 times with successive portions of reagent-grade water. Designate these as group B.

4. Wash another six Petri dishes with detergent wash water and dry without further rinsing. Designate these as group C.

5. Sterilise the Petri dishes of groups A, B and C by the usual procedure.

6. To test pre-sterilised plasticware, designate six plastic Petri dishes as group D.

7. Add 1 ml of the *E. coli* culture from step 1 to each dish and mix thoroughly with 15 ml of molten yeast-extract agar, cooled to 50 °C (nutrient agar is acceptable if yeast extract agar is not available).

8. Incubate the dishes at 37 °C for 24 hours. Count the number of colonies on each dish and calculate the mean count for each group.

9. The difference in the mean count between the groups should be less than 15 per cent if there are no toxic or inhibitory effects. Differences in mean counts of less than 15 per cent

between groups A and B, or of more than 15 per cent between groups A and C show that the detergent has inhibitory properties that are eliminated during routine washing.

10.5.3 Media

Every new batch of media should be tested for sterility and for its ability to support the growth of the test organism with the production of its characteristic biochemical reactions.

Sterility should be tested by incubating a sample volume of the medium, at an appropriate temperature, for 48 hours and observing for growth.

The ability of a medium to support the growth of a test organism and to differentiate the test organisms from other organisms is tested by inoculating a sample of the medium with positive and negative control organisms. The positive control must include a type strain of *E. coli* or a well characterised laboratory isolate. The negative control may include one or more of the following: *Staphylococcus aureus*, *Pseudomonas* sp., *Streptococcus faecalis*.

Results of the control tests should be entered in a media log-book. If the positive control does not grow or does not give the correct growth characteristics, the purity and the identification of the test culture should be checked and the test repeated with a fresh culture prepared from a frozen stock. If there are no problems with the test culture, a new batch of medium should be prepared and glassware records checked to ensure that no inhibitory substances have been found during quality control checks. The pH of the medium should also be checked, since pH changes can affect the growth characteristics of an organism. Each new batch of media should be tested alongside the media currently in use.

10.5.4 Evaluation of reagents, media and membranes

Before new batches of reagents, media, membranes and membrane pads are released for routine use, they should be compared with those currently in use. Only one variable should be changed with each comparison.

Procedure

1. Assemble at least five positive water samples (samples that have been shown to be contaminated). The use of more samples will increase the sensitivity of the test.

2. Process the samples using the test batch of materials and the batch currently in use.

3. Incubate the plates (or tubes).

4. Compare the growth characteristics of the contaminating organism on the two batches of materials. Note any atypical results.

5. Count or calculate the number of colonies per 100 ml, or determine the MPN.

6. Transform the counts to logarithms and enter the results for the two batches of materials in parallel columns.

7. Calculate the difference *d* between the two transformed results for each sample (include the + or? sign).

8. Calculate the mean of the differences $\bar{d}$ and the standard deviation.

9. Perform a Student's *t*-test, using the number of samples as *n*.

10. Use a Student's table to determine the critical value of *t* at the 0.05 significance level (two-tailed test). Some critical values are given below.

11. If the calculated value of *t* exceeds the critical value, the two batches of materials give significantly different results.

No. of samples (n)	Degrees of freedom (n B 1)	Critical value of t at 0.05 significance level
5	4	2.78
6	5	2.57
7	6	2.45
8	7	2.37
9	8	2.31
10	9	2.26

If this test indicates a problem with the new batch of materials, the test conditions and procedure should be carefully reviewed and the batch retested. The batch should be rejected as unsatisfactory only if the problems are confirmed by this second test.

10.5.5 Precision testing

Precision testing is important in the microbiological laboratory because test results can reveal procedural problems and problems with the materials. The principles of precision testing are discussed in detail in Chapter 9. Satisfactory results must be obtained from precision tests before the results of monitoring tests are reported.

Procedure

1. At the beginning of each month, or at the earliest convenient time, collect 15 samples that are likely to be positive by the first procedure, with a range of positive results.

2. Make duplicate analyses of each sample. The same analyst should do the tests, but all analysts should be included, on a rota basis.

3. Record the results of the duplicate tests as D_1 and D_2. Calculate the logarithm of each result. If either of a set of duplicate results is zero, add 1 to both values before calculating the logarithms.

4. Calculate the difference *R* between each pair of transformed duplicates, and the mean of these differences $\overline{R}$.

5. Calculate the precision criterion as 3.27 *R*.

6. Thereafter, analyse 10 per cent of routine samples, or a minimum of two samples per day, in duplicate. Calculate the logarithm of each result and the difference between the logarithms. If the difference is greater than the calculated precision criterion, analyst variability is excessive and the analytical procedure should be reviewed. The laboratory

manager should decide whether or not to release monitoring test results in the light of past performance and other mitigating factors.

10.6 Source literature and further reading

APHA 1992 *Standard Methods for the Examination of Water and Wastewater*. 18th edition, American Public Health Association (APHA), American Water Works Association (AWWA) and Water Pollution Control Federation (WPCF), Washington, D.C.

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ISO 1988 *Water Quality C Enumeration of Viable Microorganisms. Colony Count by Inoculation in or on a Solid Medium*. International Standard ISO 6222, International Organization for Standardization, Geneva.

ISO 1990a *Water Quality C Detection and Enumeration of Coliform Organisms, Thermotolerant Coliform Organisms and Presumptive* Escherichia coli. *Part 1: Membrane Filtration Method*. International Standard ISO 9308-1, International Organization for Standardization, Geneva.

ISO 1990b *Water Quality C Detection and Enumeration of Coliform Organisms, Thermotolerant Coliform Organisms and Presumptive* Escherichia coli. *Part 2: Multiple Tube (Most Probable Number) Method*. International Standard ISO 9308-2, International Organization for Standardization, Geneva.

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