Technical Assistance
HYDROLOGY PROJECT

World Bank & Government of The Netherlands funded

Training module # WQ - 22

Coliforms as Indicator of Faecal Pollution

New Delhi, June 1999

CSMRS Building, 4th Floor, Olof Palme Marg, Hauz Khas,
New Delhi – 11 00 16 India
Tel: 68 61 681 / 84 Fax: (+ 91 11) 68 61 685
E-Mail: dhvdelft@del2.vsnl.net.in

DHV Consultants BV & DELFT HYDRAULICS

with
HALCROW, TAHAL, CES, ORG & JPS

Table of contents

Page

1. Module context

This module introduces the subject of testing for bacteriological quality of water and explains how coliform bacteria can be used as indicators of pollution. Modules in which prior training is required to complete this module successfully and other related modules in this category are listed below.

While designing a training course, the relationship between this module and the others, would be maintained by keeping them close together in the syllabus and place them in a logical sequence. The actual selection of the topics and the depth of training would, of course, depend on the training needs of the participants, i.e. their knowledge level and skills performance upon the start of the course.

No.	Module title	Code	Objectives
1	Basic water quality concepts[a]	**WQ - 01**	• Become familiar with common water quality parameters • Appreciate important water quality issues
2	Basic chemistry concepts[a]	**WQ - 02**	• Convert units from one to another • Understand the basic concepts of quantitative chemistry • Report analytical results with the correct number of significant digits
3	How to prepare standard solutions	**WQ - 04**	• Recognise different types of glassware • Use an analytical balance and maintain it • Prepare standard solutions
4	Introduction to microbiology[a]	**WQ - 20**	• classify different types of micro-organisms • identify certain water borne diseases
5	Microbiological laboratory[a] techniques	**WQ - 21**	• Explain methods of bacteria identification • Discuss methods of bacteria enumeration • Follow methods of good laboratory practice
6	How to measure coliforms	**WQ - 23**	• Measure total and faecal coliforms in water samples

a- prerequisite

2. Module profile

Title	:	Coliforms as Indicator of Faecal Pollution
Target group	:	HIS function(s): Q2, Q3, Q5, Q6, Q7, Q8
Duration	:	1 session of 60 min
Objectives	:	After the training the participants will be able to*:* • Identify the main water quality problems caused by micro-organisms • Explain why coliform bacteria are good indicators • Explain the principles of the coliform analysis method
Key concepts	:	• Water borne organisms • Faecal pollution • Indicator organisms • Analysis methods
Training methods	:	Lecture, open discussion and exercises
Training tools required	:	Board, flipchart, OHS
Handouts	:	As provided in this module
Further reading and references	:	• The Microbial World, Stanier et al, Prentice-Hall, 1986 • Standard Methods: for the Examination of Water and Wastewater, APHA, AWWA, WEF/1995. APHA Publication

3. Session plan

No	Activities	Time	Tools
1	***Preparations***		
2	***Introduction***: • Explain the difficulties in testing for specific disease causing organisms • Introduce the concept of indicator organisms	10 min	OHS
3	***Coliform bacteria*** • Discuss the suitability of coliforms as indicators and describe the related groups • Faecal and non-faecal origin	10 min	OHS
4	***Coliform analysis*** • Multiple fermentation tube method • Presumptive and confirmatory tests	15 min	OHS
5	***MPN*** • Estimation from tables and formula • Examples	15 min	OHS
6	***Wrap up*** • Review material covered • Invite comments	10 min	

4. Overhead/flipchart master

OHS format guidelines

Type of text	Style	Setting
Headings:	OHS-Title	Arial 30-36, with bottom border line (not: underline)
Text:	OHS-lev1 OHS-lev2	Arial 24-26, maximum two levels
Case:		Sentence case. Avoid full text in UPPERCASE.
Italics:		Use occasionally and in a consistent way
Listings:	OHS-lev1 OHS-lev1-Numbered	Big bullets. Numbers for definite series of steps. Avoid roman numbers and letters.
Colours:		None, as these get lost in photocopying and some colours do not reproduce at all.
Formulas/Equations	OHS-Equation	Use of a table will ease horizontal alignment over more lines (columns) Use equation editor for advanced formatting only

Coliforms as Indicator of Faecal Pollution

- Water borne diseases
- Indicator bacteria
- Standards
- Coliform analysis
- MPN estimation

Water borne diseases

- Many different pathogenic micro-organisms cause waterborne disease:
  - *bacteria*
  - *viruses*
  - *protozoa*
- Many of these pathogenic micro-organisms are of faecal origin

Indicator bacteria

- Methods of measurement of pathogens can be:
  - *direct analysis for individual pathogenic organisms*
  - *analysis for indicator bacterial groups which reflect the presence of faecal contamination*
- Direct analysis of specific pathogens is costly and difficult
- Preferred method is analysis for indicator bacterial groups, e.g. coliform bacteria

Coliforms as Indicator of Faecal Pollution

- Coliform bacteria widely used internationally as indicators
- They imply presence of other pathogenic micro-organisms, specifically organisms of faecal origin

Coliform bacteria

- Coliforms are good indicator organisms because:
  - *they are abundant in faeces*
  - *they are generally found only in polluted waters*
  - *they are easily detected by simple laboratory tests*
  - *they can be detected in low concentrations in water*
  - *the number of indicator bacteria seems to be correlated with the extent of contamination*

Coliform bacteria

- Some points of caution:
  - *not ALL coliforms come from human faeces*
  - *coliforms also originate from other mammals, or birds*
  - *some coliform bacteria also naturally found in soils, water*
  - *coliform bacteria will die off in water, so even water with no coliforms may have been contaminated.*

Different groups of indicator bacteria

- Total coliforms
- Faecal coliforms
- Thermotolerant bacteria
- Faecal streptococci
- E. coli

Total coliforms

- *a group of several distinct types of bacteria*
- *Not ALL coliforms are of faecal origin*
- *some are normal inhabitants of unpolluted soils and water*
- *faecal coliform levels are ~20% of total coliforms*
- *widely used as general measure of faecal contamination*
- *identified by fermentation of lactose in 24 hrs. at 35oC*

Faecal coliforms

- *one of the groups within the 'Total coliforms'*
- *are specifically indicative of faecal contamination from humans or warm blooded animals*
- *includes the specific bacterium E. coli*
- *identified by fermentation of lactose in 24 hrs. at 44^{o}C*
- *a few non-faecal bacteria (<5%) can be indicated in this test*

Faecal streptococci

- Includes several species or varieties of streptococci
- They normally reside in intestinal tract of humans and animals
- Individual species include:
  - *Streptococci faecalis (from humans)*
  - *Streptococci bovis (from cattle)*
  - *Streptococci equinus (from horses)*

Escherichia coli

- Escherichia coli (E. coli):
  - *a specific bacterium of the faecal coliform group*
  - *resides in human intestinal tract*
  - *definitely indicates presence of faecal contamination*
  - *excreted in large numbers: ~50 million per gram*
  - *untreated domestic wastewater has 5-10 million of these coliforms per 100 ml*

Standards

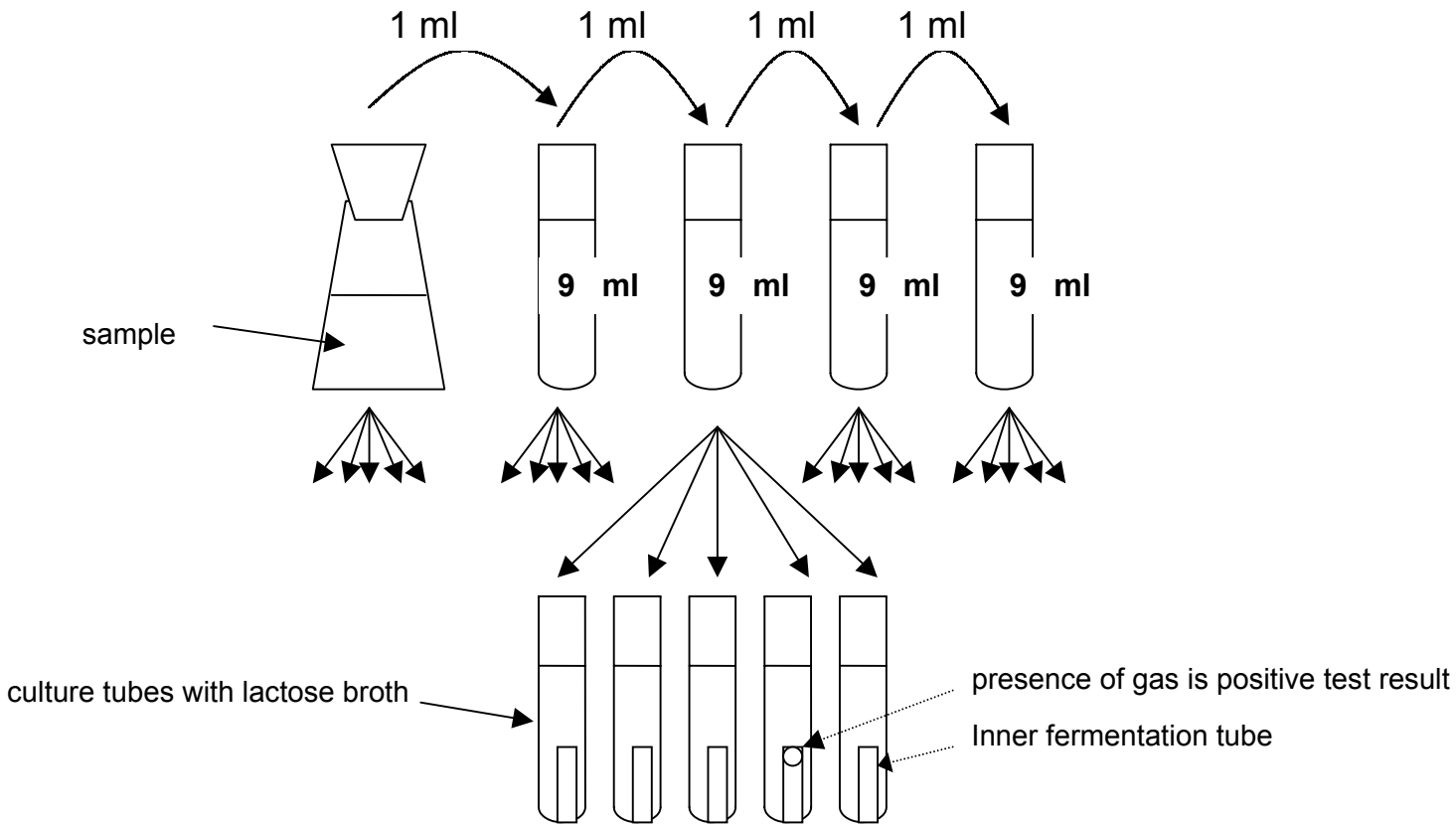
Use	Total Coliform MPN per 100 ml	Faecal Coliform MPN per 100 ml	Agency / Country
Public water supply	0	0	WHO
Drinking water source, no conventional . treatment, with disinfection	<50	no value	India
Drinking water source, with conventional treatment, and disinfection	5000	no value	India
Bathing, recreation water	5000 guide 10,000 mandatory	100 guide 2000 mandatory	Europe
Outdoor bathing (organized)	500	no value	India
Shellfishing	70	no value	US
	no value	14	Venezuela, Mexico

Coliform analysis

- *done in liquid culture of lactose broth*
- *coliform bacteria ferment lactose, producing gas*
- *common test is 'Multiple tube technique'*
- *several tubes of liquid culture are inoculated with predetermined volumes of the sample*

Coliform analysis

- Multiple tube method after a series of dilutions



Coliform analysis

- Total Coliform test includes 2 stages:

  1. presumptive test:
     - *gas production from fermentation of lactose broth at 35^{o}C*
  2. confirmed test:
     - *used to confirm a positive presumptive test*
     - *gas production in brilliant green lactose bile broth at 35^{o}C*

- The Most Probable Number (MPN) of total coliforms is calculated from the number of confirmed tubes

Coliform analysis

- Test for faecal coliforms:
- Confirmed test in EC broth at $44^{O}C$
- At the high temperature, only faecal coliform can grow and produce gas.

MPN estimation

- For the no. of positive tubes MPN values can be calculated, or looked up in standard table
- Calculation formula:

$$\text{MPN/100 mL} = \frac{\text{no. of positive tubes} \times 100}{\sqrt{\text{mL sample in negative tubes} \times \text{mL sample in all tubes}}}$$

MPN estimation from tables, examples

- Number of positive tubes out of 5

Ex. No.	10 mL	1 mL	0.1 mL	0.01mL
1	5/5	3/5	1/5	1/5
2	5/5	5/5	1/5	0/5

- **Ex. 1** Start with inoculum giving all 5 +, read, (5-3-1), MPN = 110/100mL
- **Ex. 2** Start with the highest dilution (least inoculum) giving all 5 +, read, (5-1-0), MPN = 10x30 = 300/mL

MPN estimation from tables, examples

- Number of positive tubes out of 5

Ex. No.	0.1 mL	0.01 mL	0.001 mL	0.0001mL
3	4/5	3/5	1/5	0/5
4	5/5	4/5	1/5	1/5

- **Ex. 3** Start with inoculum giving highest no. of +ive, read (4-3-1), not (3-1-0), MPN = 33x100 = 3,300/100mL
- **Ex. 4** Incorporate the +ive of the higher dilution in the highest dilution of the selected combination, read (5-4-2), not, (5-4-1), MPN = 220x100=22,000/100mL

MPN estimation using formula

$$\text{MPN/100 mL} = \frac{\text{no. of positive tubes} \times 100}{\sqrt{\text{mL sample in negative tubes} \times \text{mL sample in all tubes}}}$$

- *Count of positive tube begins with the highest dilution in which at least one negative result has occurred.*

- Ex 1: For 10, 1, 0.1 mL, positives are 5/5, 3/5, 1/5
  - *no. of positive tubes = 3 + 1 = 4, neglect 10 mL inoculum*
  - *sample in negative tubes = 2 + 0.4 = 2.4 mL*
  - *sample in all tubes = 5 + 0.5 = 5.5 mL, MPN/100mL=110*

5. Evaluation sheets

6. Handout

Coliforms as Indicator of Faecal Pollution

- Water borne diseases
- Indicator bacteria
- Standards
- Coliform analysis
- MPN estimation

Water borne diseases

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- *Thermotolerant bacteria*
- *Faecal streptococci*
- *E. coli*

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  - *sample in all tubes = 5 + 0.5 = 5.5 mL, MPN/100mL=110*

Add copy of Main text in chapter 8, for all participants.

7. Additional handout

These handouts are distributed during delivery and contain test questions, answers to questions, special worksheets, optional information, and other matters you would not like to be seen in the regular handouts.

It is a good practice to pre-punch these additional handouts, so the participants can easily insert them in the main handout folder.

8. Main text

Contents

Coliforms as Indicator of Faecal Pollution

1. Introduction

The problem of waterborne diseases is relevant in many countries particularly the developing countries. The impact of high concentrations of disease-producing organisms on water users can be significant.

Disease producing organisms are also known as 'pathogens'. Pathogenic organisms can generally be classified as:

- pathogenic bacteria
- viruses
- parasites (e.g. protozoa and intestinal worms (helminths))

Some examples are listed in Table 1.

Table 1 Classification of pathogenic micro-organisms

Type	***Examples***	***Diseases***
Pathogenic bacteria	*Vibrio cholerae*	cholera
	Salmonella species	typhoid
	Shigella species	dysentery
Viruses	Hepatitis A	hepatitis
	Polio viruses	polio
	Enteroviruses Echoviruses	central nervous system disorders
Intestinal parasites (protozoa and intestinal worms – helminths)	*Giardia lambia*	giardiasis (diarrhoeal disease)
	Entamoeba histolytica	amoebic dysentery
	Facultatively parasitic amoebae (*Naegloria* and *Hartmanella)* Helminths (e.g. whipworm, hookworm, dwarf tapeworm)	

Many, but not all, of these pathogenic organisms are of faecal origin, The method of transmission of pathogens is through ingestion of contaminated water and food, and exposure to infected persons or animals. Infections of the skin, eys, ears, nose and throat may result from immersion in water while bathing. Specific modes of infection are:

- drinking water: municipal, domestic, industrial and individual supplies
- direct (primary) contact with polluted water: bathing
- secondary contact with polluted water: boating, fishing, clothes washing
- eating fish / shellfish

Methods of measurement of bacteriological quality can be:

- direct analysis for pathogenic bacteria
- analysis for viruses
- analysis for intestinal parasites
- analysis for **indicator bacterial groups** which reflect the potential presence of pathogens, e.g. coliform bacteria

The determination of specific pathogenic bacteria, viruses, or parasites requires a high degree of expertise, especially when they are present in low numbers. The use of indicator bacterial groups has always been a favoured method.

2. Indicator Bacteria

The group of coliform bacteria as an indicator of other pathogenic micro-organisms, specifically organisms of faecal origin, has had much emphasis in all countries. This is due primarily to the fact that the coliform bacteria group meet many of the criteria for a suitable indicator organism, and are thus a sensitive indicator of faecal pollution:

- they are abundant in faeces
- they are generally found only in polluted waters,
- they are easily detected by simple laboratory tests,
- can be detected in low concentrations in water
- the number of indicator bacteria seems to be correlated with the extent of contamination.

It is important to remember, however, that not all coliforms emanate from human faeces as they can originate from other mammalian species or from other environmental sources (e.g., bird droppings).

When coliforms are discharged to the aquatic environment they will tend to die at a rate which depends, amongst other things, on the temperature and turbidity of the water and the depth to which solar radiation penetrates. Therefore, it is not safe to conclude that the lack of coliforms in a water means that it has not been subject to faecal pollution.

Text books of microbiological analysis use differing terminology to refer to the coliform group of organisms. For this reason, it is necessary to be familiar with a number of terms which may be used in this context as follows:

Total coliforms
The Total coliform group comprises several distinct types (genera) of bacteria. These bacteria have been isolated from the faeces of humans and other warm-blooded animals, as well as contaminated and non-contaminated soils. This group of bacteria is widely used as a measure of health hazard from faecal contamination. The total coliform group comprises the aerobic and facultative, gram negative, nonspore-forming, rod shaped bacteria that ferment lactose with gas formation within 48 hours at 35 °C.

Faecal coliforms
The Faecal coliform group of bacteria are indicative of faeces of humans and other warm blooded animals. The specific bacterium *Escherichia coli* is part of this group. The test for faecal coliform is at an elevated temperature, 44.5 °C, where growth of other non-faecal bacteria is suppressed. However, some non-faecal bacteria may be also be identified in the faecal coliform test, though a small percentage (<5%)).

Faecal streptococci
This group of bacteria includes several species or varieties of streptococci and the normal habitat of these bacteria is the intestines of humans and animals. Examples include *Streptococci faecalis* which represents bacteria of humans and *Streptococci bovis* and *Streptococci equinus* which represent bacteria that are indicators of cattle and horses.

Thermotolerant coliforms
This is a more precise definition of coliforms which are determined by the test for faecal coliforms. In practise not all such coliforms are faecal in origin although most (> 95%) are.

Escherichia coli (E. coli)
This bacterium is a particular member of the faecal coliform group of bacteria; this organism in water indicates the presence of faecal contamination.

E. coli reside in human intestinal tracts. They are excreted in large numbers in faeces, averaging about 50 million per gram. Untreated domestic wastewater generally contains 5 to 10 million coliforms per 100 ml.

Pathogenic bacteria and viruses causing enteric diseases in humans originate from faecal discharges of diseased persons. Consequently, water containing coliform bacteria is identified as potentially dangerous.

Coliform bacteria are, therefore, considered as an indicator of bacteriological quality of water for the following reasons:

- coliform bacteria far out number the pathogenic micro-organisms,
- they do not multiply in natural waters,
- the die-off rate of pathogenic bacteria is greater than the death rate of coliforms
- test for Coliform bacteria is relatively simple and can be performed in water quality laboratories

The bacterium *E.coli* is exclusively of faecal origin. Some coliform bacteria are normal inhabitants of soil and water. In testing for conforms, therefore, tests may be run in conjunction to verify their faecal origin. However, unconfirmed testing, indeed, would provide a factor of safety.

The degree to which indicator organisms represent the presence of individual pathogens (such as Salmonella) has been the subject of continuing investigation. There does seem to be a genera correlation between the concentration of Faecal coliform bacteria and the occurrence of *Salmonella*. When faecal coliform numbers are about 1000 per 100 ml, *Salmonella* occurrence is about 95 %

Relationships between total coliform and individual pathogens is not so quantitative. Thus the test of total coliform is not so effective for an indicator. The total coliform test is complicated by the presence of non-faecal bacteria. As a general rule, faecal coliform levels are about 20% of total coliform concentrations, although a wide spread exists.

3. Bacterial Standards

Many countries or international organisations have water quality standards for bacteria. A few are given in Table 2.

Table 2 Some Indian and international water quality standards for indicator bacteria groups.

Use	Total Coliform MPN per 100 ml	Faecal Coliform MPN per 100 ml	Agency / Country
Public water supply	0	0	WHO
Drinking water source, no conventional . treatment, with disinfection	<50	no value	India
Drinking water source, with conventional treatment, and disinfection	5000	no value	India
Bathing, recreation water	5000 guide 10,000 mandatory	100 guide 2000 mandatory	Europe
Outdoor bathing (organized)	500	no value	India
Shellfishing	70	no value	US
	no value	14	Venezuela, Mexico

4. Organism Decay Rate

Once pathogenic organisms are in surface water, their survival and fate depends on a number of factors such as :

- sunlight
- temperature
- salinity
- predation
- nutrient conditions
- toxic substances
- settling and resuspension
- continued growth

Typically, the pathogenic organisms will not reproduce in surface waters, and will die following an exponential first-order decay curve.

$$C = C_o e^{-Kbt}$$

where:

Co = the original concentration of bacteria
C = the concentration of bacteria after time (t)
Kb = bacterial decay constant (day^{-1})
t = time (day)

A common manner of expressing the decay of bacteria is the T90 coefficient. T90 is the time needed to obtain 90% mortality of the original number of bacteria assuming a first-order decay.

$$\frac{C}{C_o} = 0.10 = e^{-Kb\,(T\,90)}$$

Solving:

$$\ln (0.1) = -Kb\ (T90)$$

$$\Rightarrow \quad 2.3 = Kb\ (T90)$$

$$\Rightarrow \quad T90 = 2.3 / Kb$$

Values of the decay coefficient (Kb)

Decay rates increase with increasing temperature, sunlight, and salinity.

Table 3 Typical ranges of decay coefficients for different pathogens

Organism	**Freshwater Kb (day^{-1})**	**Seawater Kb (day^{-1})**
Total coliform	1 –84	0.8
E. Coli	0.087-3.0	0.7-3.0
Faecal coliform		
Feacal streptococci	0.5 - 3	18 - 55
Salmonella	0.5 – 3	
Viruses	0.03 – 0.8	1.1 - 2.3

5. Coliform Analysis

The test for coliform bacteria is usually conducted using a liquid culture. Enumeration employing solid culture media is not commonly done in India. The liquid culture 'multiple tube technique' consists of 2 stages:

1. presumptive test
2. confirmed test

The presumptive test is based on gas production during fermentation of lauryl tryptose broth which contains beef extract, peptone and lactose within 48 hour of incubation at 35^{o}C.

The confirmed test is used to substantiate or deny the presence of coliforms in a positive presumptive test. A small inoculum from a positive lactose broth is transferred to a tube containing brilliant green lactose bile broth. The green dye and bile salts in this broth inhibit non- coliform growth. The presence of coliform is confirmed by growth and gas production within 48 hour at 35^{o}C. The Most Probable Number (MPN) of coliform is then calculated from the number of confirmed tubes.

Faecal coliform test

Sometimes a 'completed test' may be performed to determine the faecal origin of the coliforms giving positive confirmative test. These tests involve subculturing of the positive tubes on solid media and testing for further bio-chemical reactions.

Elevated temperature test for the separation of organisms of coliform group into those of fecal and nonfecal origin may also be performed. In this test, transfers from all positive presumptive tubes are made to culture tubes of EC medium which contains bile salts and sodium chloride as selective agents along with nutrients. The inoculated tubes are incubated at 44.5 ± 0.2 ^{O}C. Gas production within 24 hour is considered a positive reaction indicating coliforms of faecal origin.

Methods of Analysis

There are two basic analyses which can be performed to determine the presence of coliform bacteria. These are the 'multiple tube' technique and the 'membrane filter' method. A comparison of the two methods is given in Table 4 below.

Table 4 Comparison of coliform analysis methods

Multiple Fermentation Tube Method	***Membrane Filter Method***
Slower: requires 48 hours for a positive or presumptive positive	More rapid: quantitative results in 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
Low precision	High precision
Difficult to use in the field	Can be adapted for field use
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	

(Adapted from Water Quality Monitoring - a practical guide to the design and implementation of freshwater quality studies and monitoring programmes, Edited by Bartram J and Ballance R, E & F N Spon, London, 1996)

Due to the fact that equipment and consumables are more costly and sometimes more difficult to obtain in India, this training programme (and the overall project) will only use the multiple tube for coliform analysis. The membrane filtration technique will not be discussed further, therefore.

Multiple Tube Method

As referred to above, the multiple tube technique is applicable to many different water samples including those obtained from potable, fresh, brackish and salt waters. The test can also be used for the estimation of coliform bacteria in muds, sediments and sludges.

The method, which has been successfully used in many countries for the analysis of drinking and other waters, reports coliform results in terms of the 'most probable number' (MPN) of organisms. That is, the test gives the most likely number of coliform bacteria rather than the actual number.

The basis of the test is that multiple tubes of culture medium are inoculated with various dilutions of a water sample and incubated at a constant temperature for a given period of time. If coliforms are present in a tube this is detected by growth within the tube and the production of gas. Any gas produced is collected in an inverted gas collection tube placed within a larger test tube containing the culture medium. The result of the analysis, in terms of the most probable number of coliforms, depends upon the number of tubes which show a positive reaction.

Typically, the MPN value is determined from the number of positive tests in a series of 5 replicates made from 3 different dilutions or inoculation amounts (15 samples altogether). For example, sample inoculation amounts may be 10, 1 and 0.1 ml per test tube. The test method can be described as follows:

For drinking water, high numbers of coliform bacteria are not expected, so there is no need to make dilutions. Transfer a 10 ml sample into each of 10 test tubes containing a lactose culture medium and an inverted gas collection tube. MPN results can be read from Table 6.

For non-potable water, rivers, open wells and tanks transfer a 10 ml, 1 ml and 0.1 ml sample into 5 test tubes each (i.e. a total of 15 tubes). MPN results can be read from Table 5.

For non-potable and polluted waters, smaller volumes, i.e. 1 ml, 0.1 ml and 0.01 ml sample should be transferred into 5 test tubes each (i.e. a total of 15 tubes). Transferring small sample amounts is difficult, so first a series of dilutions is made . The next step is to transfer a 1 ml sample from each dilution to each of 5 test tubes containing a lactose culture medium and an inverted gas collection tube. The MPN value is read again from Table 6 and the result is multiplied by the dilution factor.

In each case, the inoculated tubes are incubated in an incubator or a water bath at 35 $\pm$ 0.5^{o}C for 24 $\pm$ 2hours. The accumulation of gas in the inverted gas-collection tubes after 24 hours is considered to be a positive presumptive test for total coliform bacteria. The number of positive tubes is confirmed as described earlier.

For the combination of positive tubes not appearing in Table 6, or in case the table is not available, the following formula is used:

$$\text{MPN/100 mL} = \frac{\text{no. of positive tubes} \times 100}{\sqrt{\text{mL sample in negative tubes} \times \text{mL sample in all tubes}}}$$

When using the above equation, remember that the count of positive tubes starts with the highest dilution in which at least one negative result has occurred.

When more than three test dilutions are incubated, the following rules are used in determining MPN value:

- Choose the highest dilution that gives positive results in all five portions tested or the largest number of positives and the two next higher dilutions.
- Where positive results occur in dilutions higher than the three chosen according to the above rule, they are incorporated in the results of the highest chosen dilution up to a total of five.
- If only one dilution gives a positive result, two dilutions immediately lower and higher giving zero positives should be chosen so as to keep the positive result in the middle of the series.

Table 5 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	2.1	16.8
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

Some examples for estimation of MPN for various cases are given in Table 7. The selected combinations are shaded. Calculations are explained below:

- Ex. 1 Regular reading of Table 6
- Ex. 2 Unusual combination, formula calculation: 1200/ (12x55.5)1/2 = 47
- Ex. 3 Adjust the selected positive tube set as 5-3-3, followed by regular reading of Table 6 x 100
- Ex. 4. Regular reading of Table 6 x 1000
- Ex. 5 Adjust the selected set as 5-0-5, which is unusual combination, formula calculation: $500/ (0.5x0.55)^{1/2}$=953
- Ex. 6 Adjust the selected positive tube set as 5-4-4, followed by regular reading of Table 6 x 100
- Ex. 7 Regular reading of Table 6 x 100
- Ex. 8 Regular reading of Table 6 x 10
- Ex. 9 Regular reading of Table 6

- Ex. 10 Adjust the selected positive tube set as 5-3-2, followed by regular reading of Table 6 x 10

Table 6. **MPN Index and 95% Confidence Limits for Various Combinations of Positive Results with Five Tubes per Dilution (10 mL, 1.0 mL, 0.1 mL)**

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

Combination of Positives	MPN Index / 100 mL	95% Confidence Limits (Approximate)	
		Lower	Upper
5-5-4	1600	600	5300
5-5-5	≥ 1600	-	-

Table 7 Examples for reading and calculating MPN values.

Ex.No	10 mL	1 mL	0.1 mL	0.01 mL	0.001 mL	0.0001 mL	MPN index /100mL	MPN /100mL
1.	5/5	3/5	1/5	-	-	-	110	110
2.	4/5	3/5	5/5	-	-	-		46
3.	5/5	5/5	5/5	3/5	2/5	1/5	170	17000
4.	5/5	5/5	5/5	5/5	3/5	2/5	140	140000
5.	5/5	5/5	0/5	3/5	2/5	1/5		953
6.	5/5	5/5	5/5	4/5	3/5	1/5	350	35000
7.	-	5/5	5/5	2/5	0/5	-	50	5000
8.	-	5/5	4/5	2/5	0/5	-	220	2200
9.	0/5	1/5	0/5	0/5			2	2
10.	-	5/5	3/5	1/5	1/5	-	140	1400