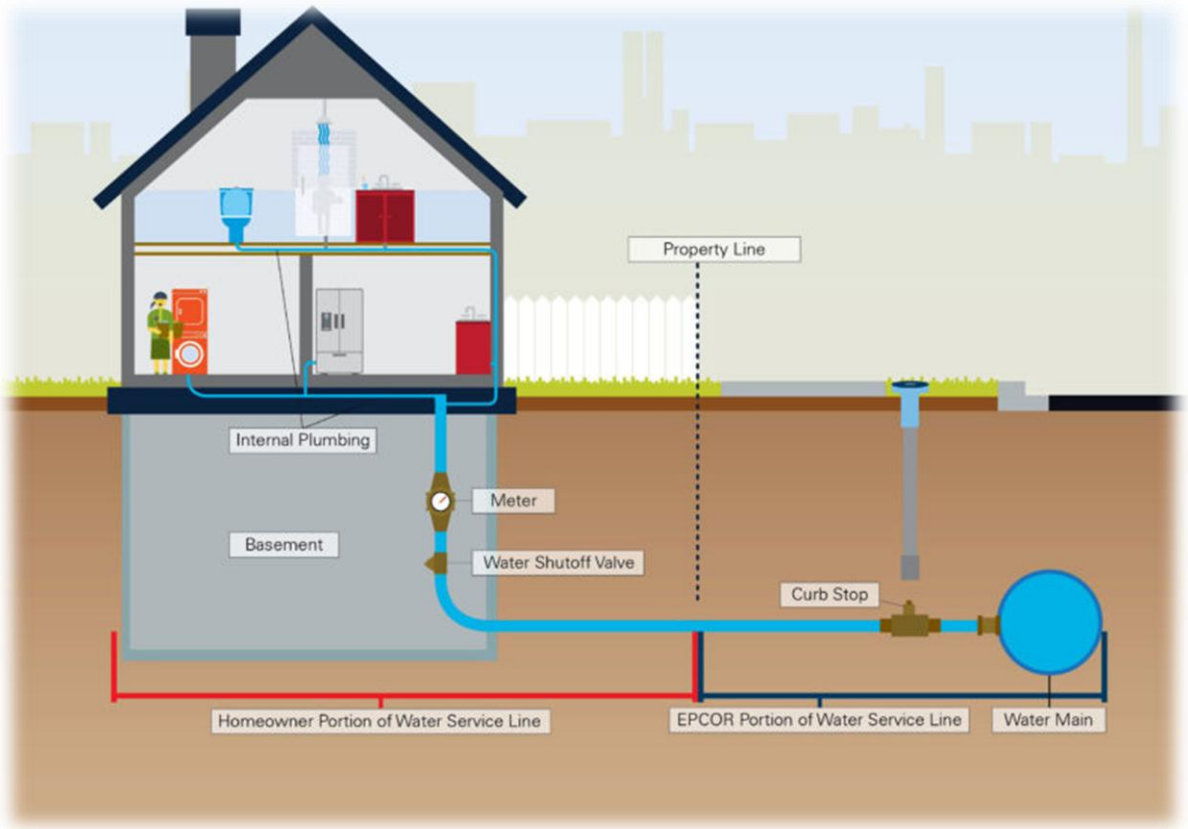
CE 4402

Environmental Engineering-II Sessional



Depatment of Civil Engineering

University of Global Village (UGV), Barishal

Preface

Environmental engineering plays a critical role in safeguarding public health and ensuring the sustainability of natural resources. One of the core responsibilities in this field is the assessment and management of microbial contamination in water systems. This sessional course, Environmental Engineering 2, is specifically designed to equip students with the essential knowledge and practical skills required to detect and evaluate microbial pollutants in various water and wastewater sources.

In this manual, emphasis is placed on both field-based and laboratory-based approaches to analyzing microbiological quality. The practical sessions introduce students to systematic procedures for collecting representative samples from different environments, handling and transporting them with care to preserve microbial integrity, and applying standard techniques to identify potential microbial threats. These activities allow students to engage with real-world challenges in water quality monitoring and help build a strong foundation in public health-related environmental practices.

The manual also highlights the importance of recognizing microbial indicators that reflect the sanitary quality of water. Through the analysis of these indicators, students can draw meaningful conclusions about the effectiveness of treatment systems, the potential for disease transmission, and the overall safety of water for various uses. By developing a critical understanding of these concepts, students will be better prepared to address environmental challenges through informed engineering solutions.

Additionally, the course fosters analytical thinking, attention to detail, and adherence to international standards and local environmental regulations. All procedures outlined in this manual are based on globally recognized methodologies and adapted to meet the requirements of field and laboratory settings commonly encountered in environmental monitoring.

By the end of this sessional course, students are expected not only to master technical procedures but also to appreciate the broader implications of microbiological contamination in water systems. This practical exposure will serve as a stepping stone for future environmental professionals working in water treatment, public health, sanitation, and regulatory compliance.

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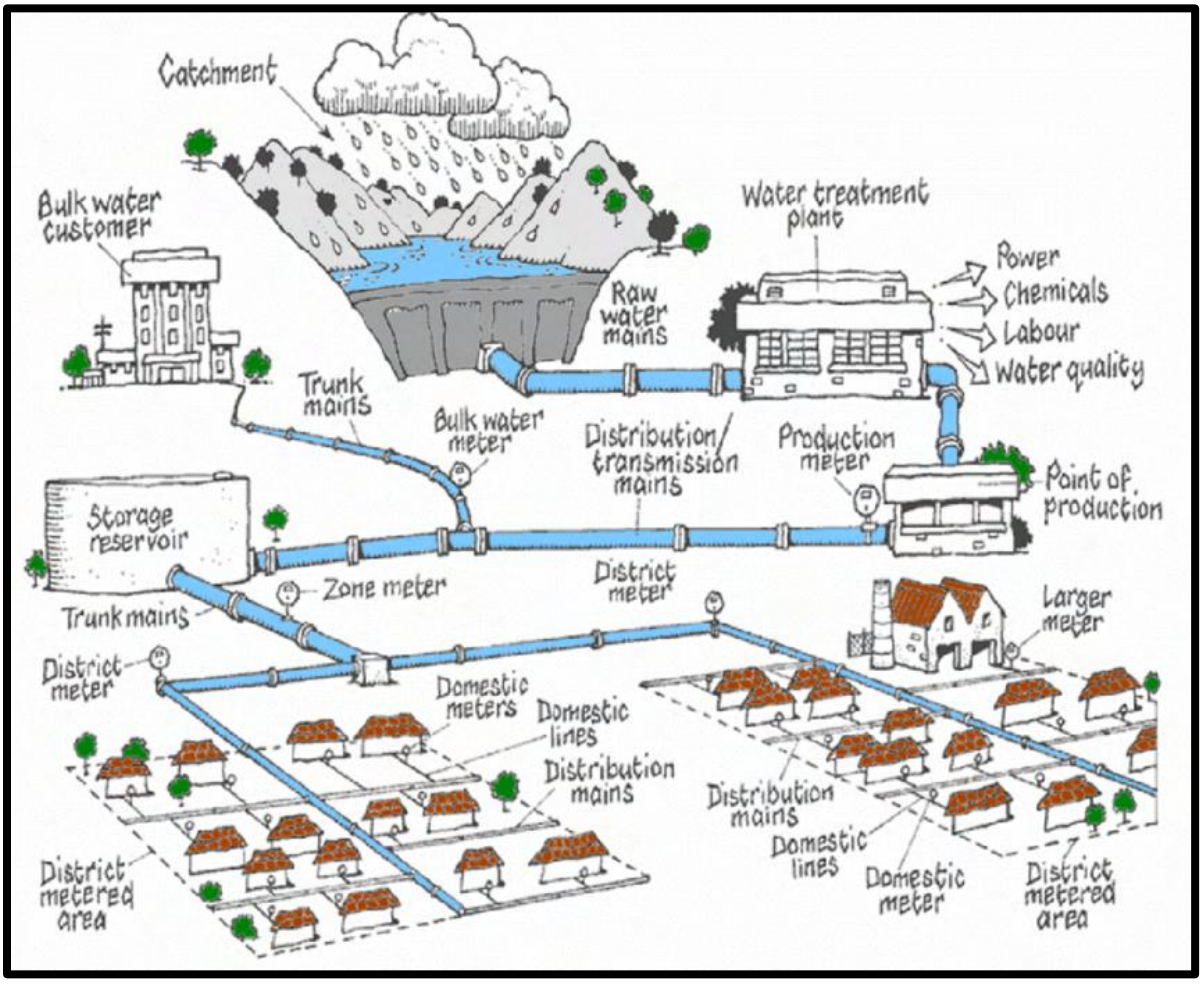
Table of Content

Experiment No. 01

Design of Water Supply System (WSS)



Purposes of Water Supply System Design

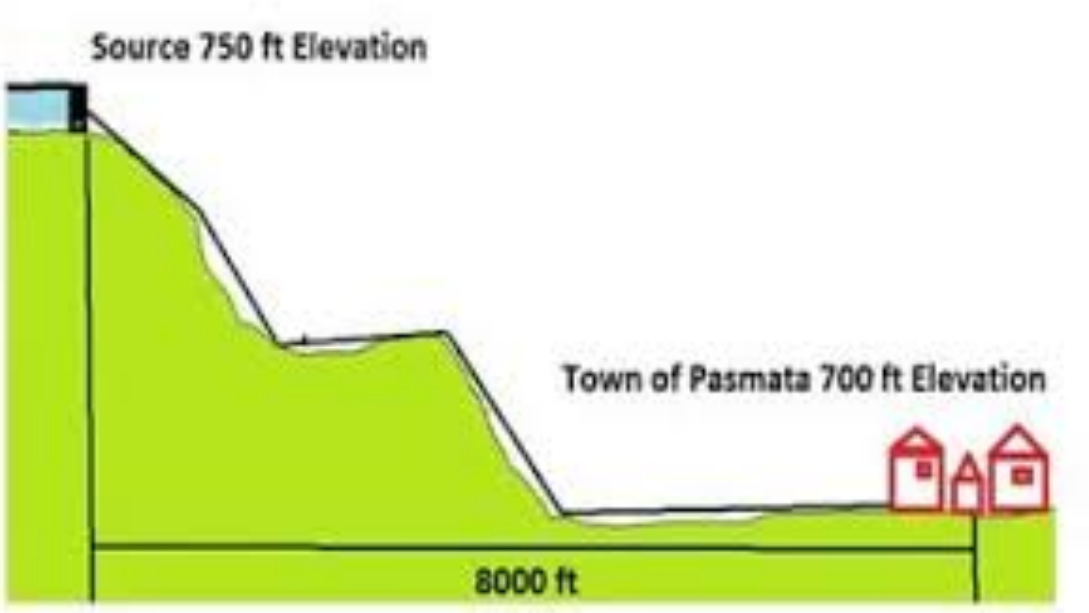
- To make water available in close proximity to the consumers
- To supply water in adequate quantities according to the demand of the consumers
- To supply water with adequate pressure
- To regulate water supply as per requirement

Classification of WSS

- Gravity flow system
- System with direct pumping
- System with pumping and storage

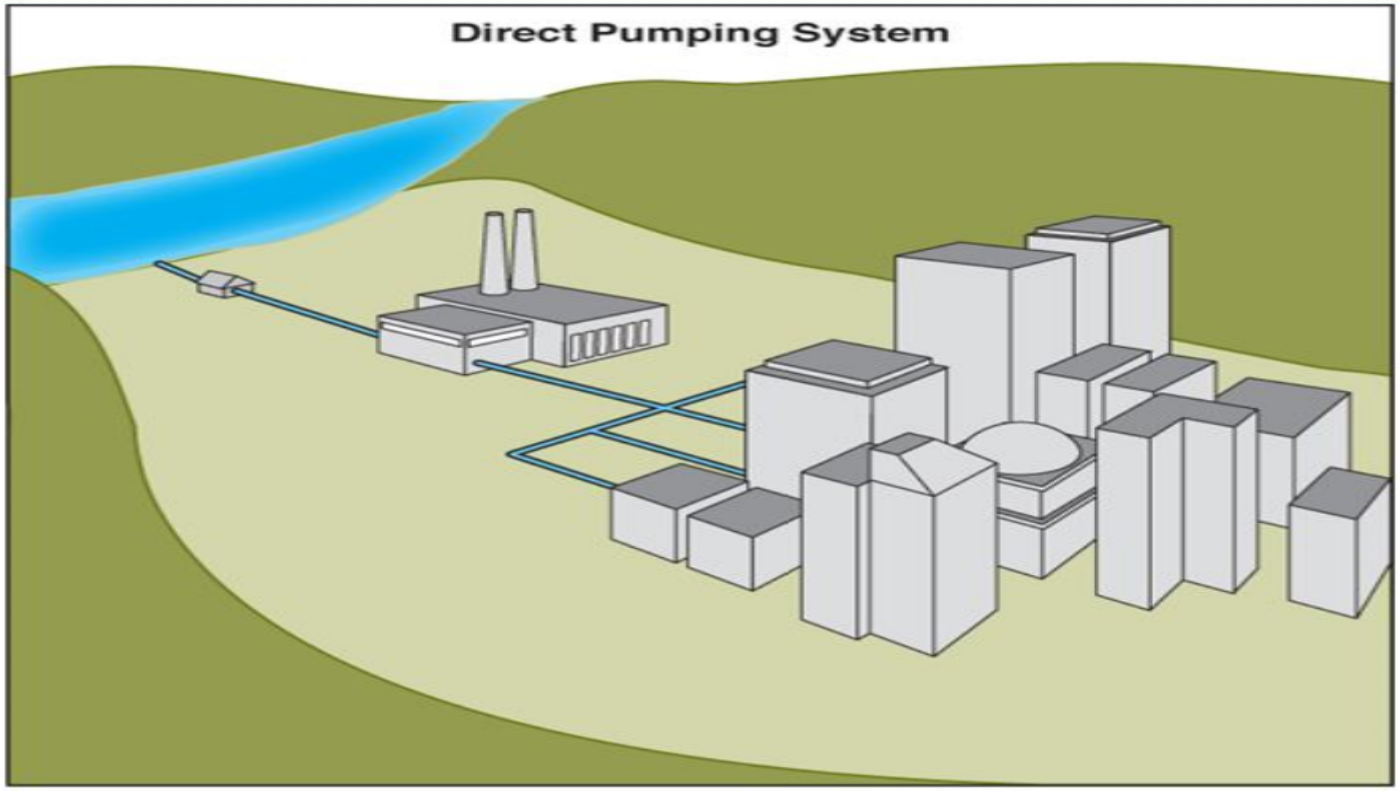
Classification of WSS

- Gravity flow system



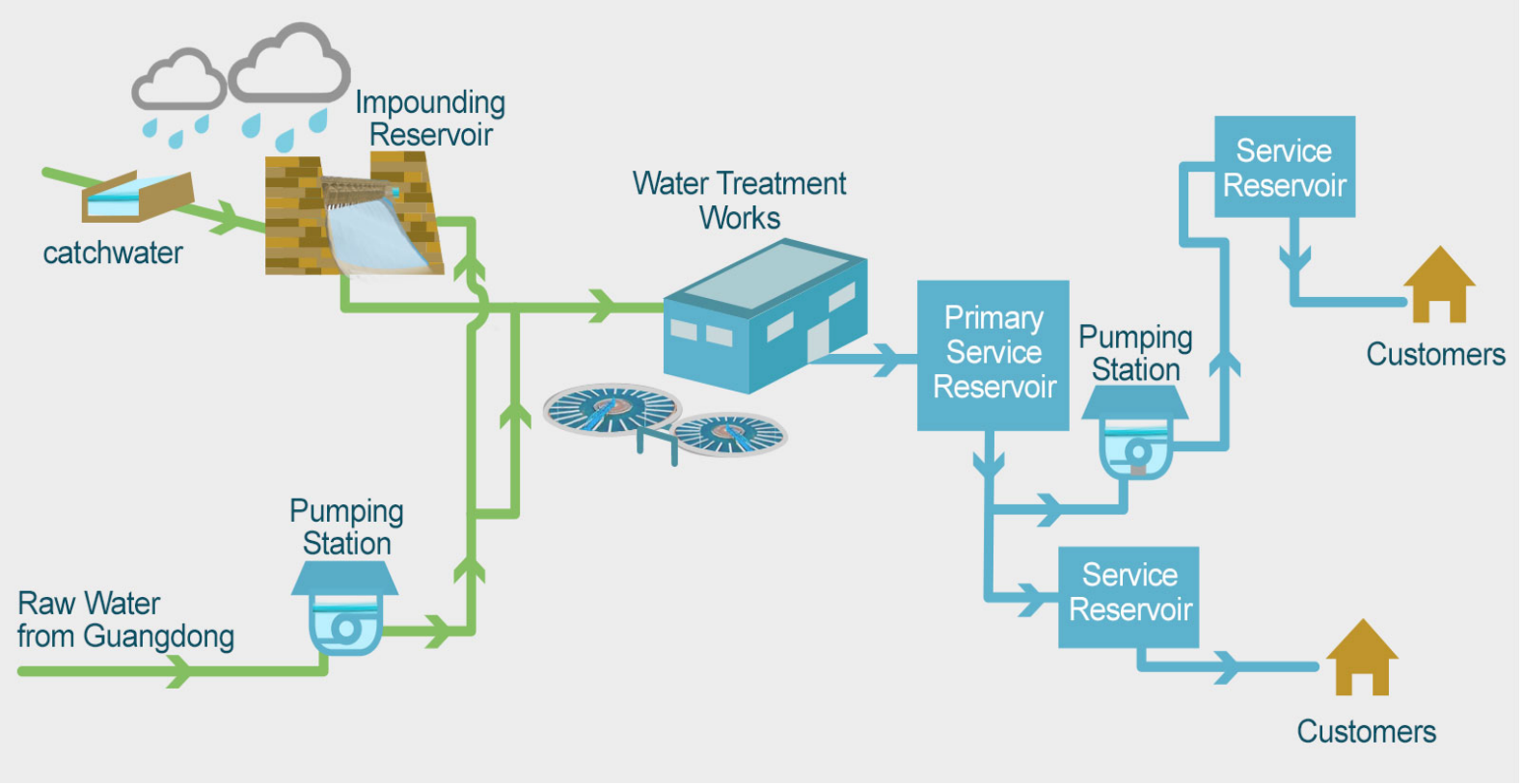
Classification of WSS

- System with direct pumping



Classification of WSS

- System with pumping and storage

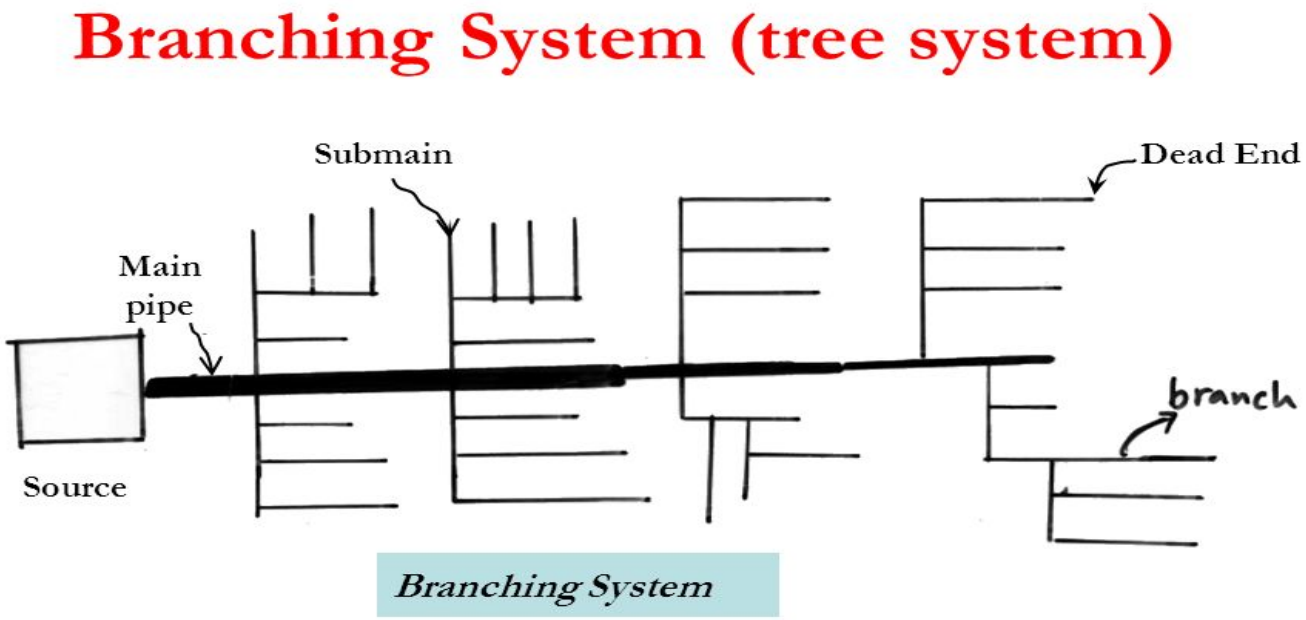


Distribution of WSS Network

- Branched distribution network
- Looped distribution network

Distribution of WSS Network

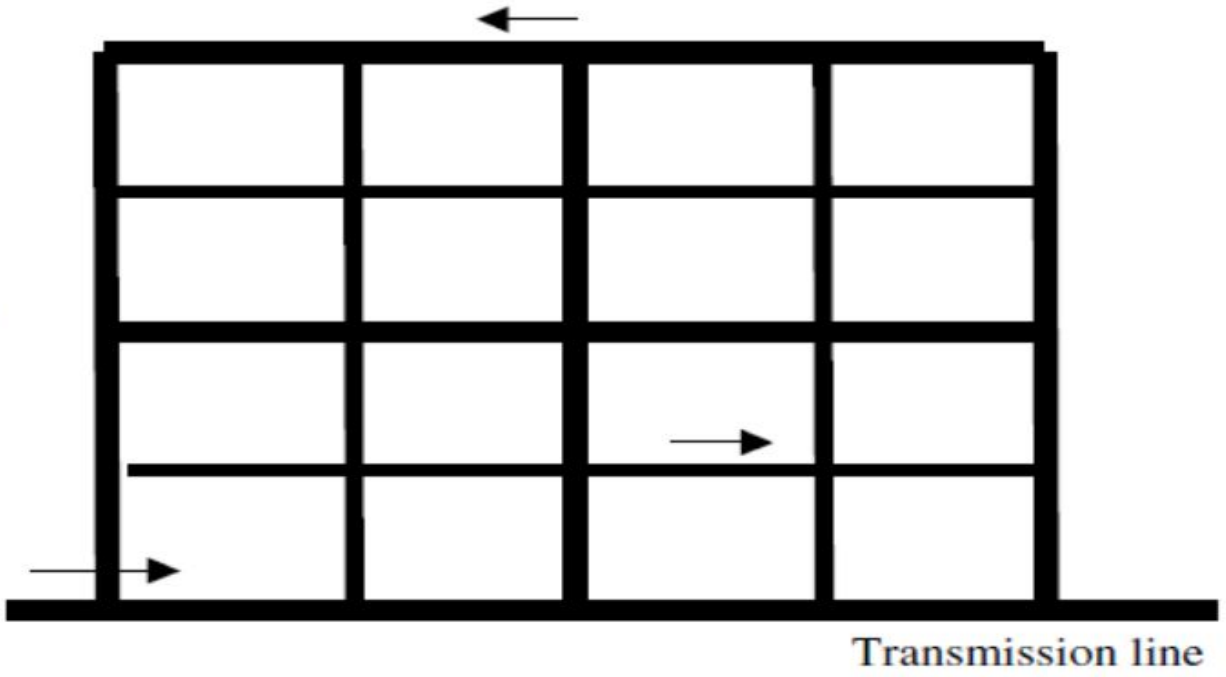
- Branched distribution network



Suitable for developing areas with an irregular pattern of road network

Distribution of WSS Network

- Looped distribution network



Suitable for well-planned developed areas with a definite pattern of road network

WSS Design Consideration

Peak factor: Daily peak factor varies from 1.3-1.5

Water consumption per capita per day in Bangladesh:

Areas	Water consumption (lpcd)	Peak factor, k
Rural areas	50	3
Upazila towns	100	2
Zilla towns	120	2
City corporations	180	1.5

WSS Design Consideration

Design flow:

$$Q = f\ q\ P_f / (1\text{-}0.01\ w)$$

Here,
Q = peak design flow per day
P_f = design population
f = peak factor
q = average water consumption per capita per day
W = loss and wastage of water in percent

Future design population:

$$P_f = P_p\ (1 + r)^n$$

Here, P_p = present population, n = design periods, r = annual growth rate of population

WSS Design

Hazen Williams Equation:

$$Q = 3.7 \times 10^{-6} \, C \, D^{2.63} \, (H/L)^{0.54}$$

Here,
Q = flows, lps
C = roughness coefficient (ranges from 100 – 140)
D = diameter of pipe, mm
H = head loss, m
L = length of pipe, m

For C = 130, $H/L = 1.39 \times 10^{6} \, Q^{1.85} / D^{4.87}$

For C = 120, $H/L = 1.59 \times 10^{6} \, Q^{1.85} / D^{4.87}$

Branched Network System Design

Design Procedure:

1. Collect/prepare a map of the area to be served with roads, streets and other features. Then make a layout of mains, sub-mains and branches including location of valves and other appurtenances.

2. Estimate the peak flow at different points. Peak flow = average daily flow x peak factor

3. Assume pipe sizes of all the pipes in the network. Assume velocity to be around 1 m/s.

4. Calculate frictional head loss per unit length of pipe using Hazen William's equation and then multiply by the pipe length to find the total head loss.

5. Determine the terminal pressure head taking the change in the elevation of the pipe into account.

6. In case of a difference between the computed terminal pressure and the permissible pressure head, revise the pipe size.

Branched Network System Design

Pipe Sizes:

Supply line	Pipe dia	Length
Main line	100 mm – 150 mm	400 m
Sub-main line	50 mm – 75 mm	200 m
Branch line	25 mm – 50 mm	100 m

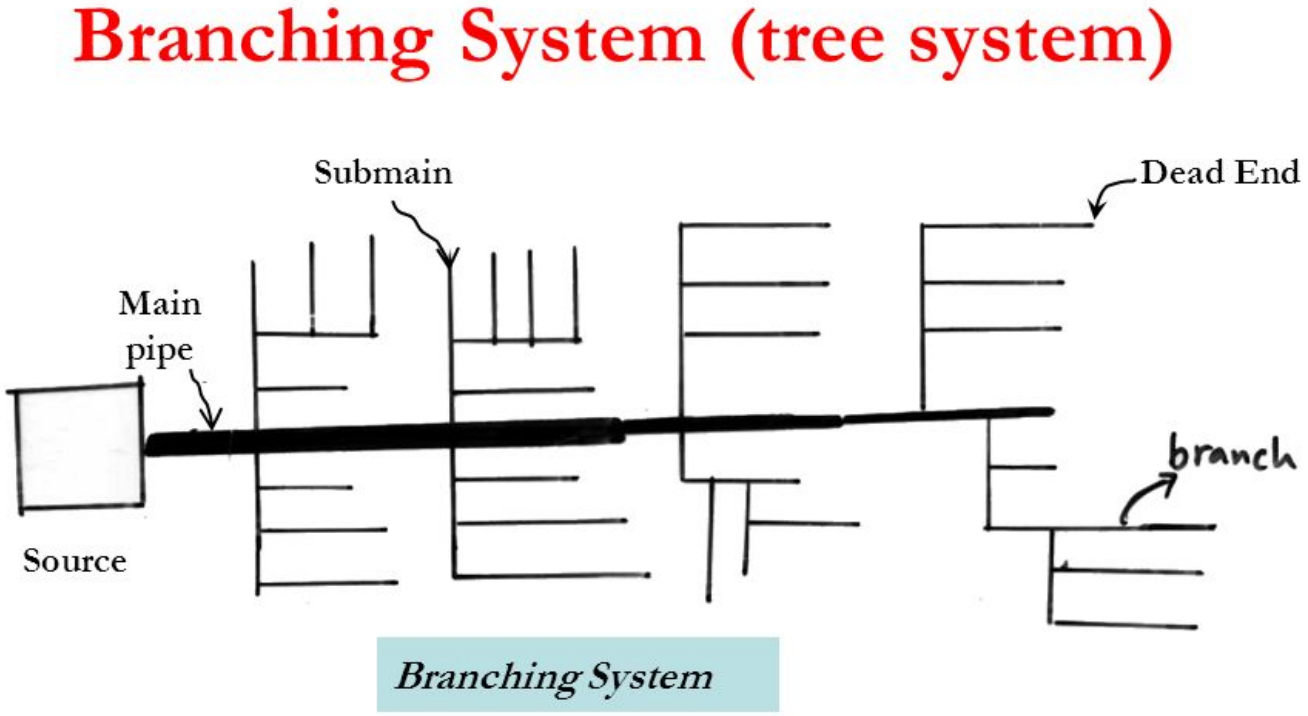
Branched Network System Design

Design Specifications:

- **Design velocity = 1 m/s**
- **Required pressure head = 18 m**
- **Supply pressure head = 25 m**
- **Annual growth rate = 3%**
- **Roughness coefficient = 120**
- **Average daily flow = 130+last two digit of Roll No. (lpcd)**
- **Design period = 25 years**
- **Peak factor = 1.5**
- **Assume, no wastage of water**

Branch Network Design

- Branched distribution network



Branched Network System Design

Design Calculation:

Main	Sub-main	Branch	Pp	Pf	Q	D	H_L	$P_T = H_A - H_L$	Check, $P_T > H_R$	Remarks
M1	SM1	B11								OK
		B12								
	SM2	B21								

Any Question?

Thank you

Experiment No. 02

Sampling of Water and Waste Water for Bacterial Test

Experiment 02

SAMPLING OF WATER AND WASTEWATER FOR BACTERIAL TEST

Bottles

- Sample should be collected in suitable bottle that have been carefully cleaned, rinsed with distilled water and sterilized.
- Bottle may be two types: (i) clean sterile bottles and (ii) clean sterile sodium thiosulfate-treated bottles.
- Sample containing residual chlorine should always be collected in sodium thiosulfate-treated bottles.
- Bottle size should be from 4 to 8 oz.
- The stopper and neck of the sample bottle should be covered to protect against dust and handling contacts.

Collection of samples

- Extreme care should be exercised to avoid contaminating parts of the bottle coming in contact with the water.
- The stopper should be handled without removal of the protective cover.
- Bottles should be filled to three-quarters of their capacity.
- If samples are collected in sodium thiosulfate-treated bottles, care must be exercised not to rinse the bottle and lose the sodium thiosulfate.

1. Sample from water systems

- The tap and piping should be thoroughly flushed before collecting the sample.
- Water should be collected from the taps that are in frequent use.
- Avoid sampling from taps, which are subject to contamination, for example, taps in lavatories.
- Avoid sampling from wet taps.
- Never sample from rubber hose or any other temporary attachment fastened to the tap.

2. Samples from Pools, Lakes Rivers

- Care should be exercised to obtain the sample from a point which represents average conditions of the supply.
- In all cases where samples are collected from standing water, remove the stopper aseptically and plunge the bottle beneath the surface, mouth down, to a depth of 3 in. or more.
- Fill the bottle as moving forward away from the hand so that water, which has come in contact with the hand, will not enter the bottle.
- Discard about a quarter of the water and replace the stopper.

Pools

- The sample should be collected at the side of the pool at a point near the deepest part.
- Sample should be collected during periods of use, preferably at the time of the heaviest bathing load of the day.
- Samples should never be taken in the absence of bathers.
- Sample should be collected in sodium thiosulfate-treated bottles.

Lakes

- Sample should never be collected from the shore.
- Sample should be collected at a distance from the shore of at least 25 ft. or more, depending upon the depth.

Rivers

- In a straight stretch of the river, sample may be collected from the bank but at a distance of at least 4 ft.
- In a meandering stream the samples should be collected near the center at the point of greatest depth.

3. Samples of sewage and sewage effluents

- The sample should always be collected directly from the sewage or sewage effluent into the sample bottle.
- Never collect the sample by means of a common collecting container.
- When composite samples are desired, samples should be collected in sterile container.
- The composite sample bottle should be stored at a temperature of 6^{o} to 10^{o}C during the period of collection.

Transportation and Storage of Samples

- Owing to biological changes that may occur in the sample of water or sewage, all samples should be tested as soon as possible.
- In warm weather, if the transportation period exceeds more that 1 hr., the sample should be iced.
- The time of transportation and storage should not exceeds 6 hr. for impure waters and not more than 12 hr. for relatively pure waters.
- Samples should be stored at a temperature between 6^{0} and 10^{0}C.
- Samples stored longer than 24 hr. should be discarded or the results obtained should be judged accordingly.

Experiment No. 03

Field Test Method for Thermotolerant Coliform

Field Test Method for Thermotolerant Coliform

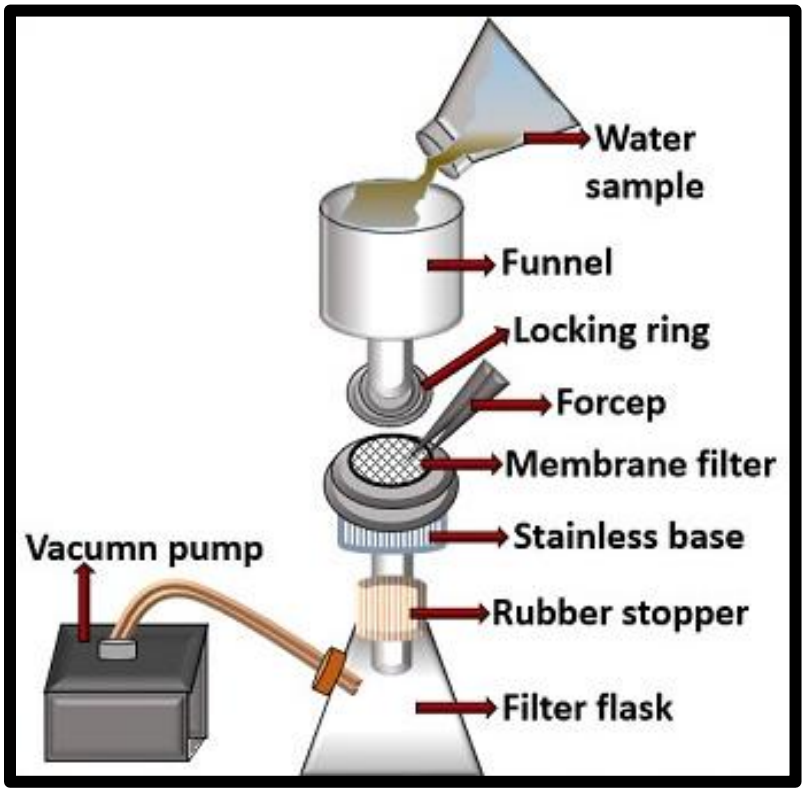
[*Courtesy: WHO Guideline for Drinking Water Quality, 2nd Edition, Volume-3, 1997*]

The field test method for thermotolerant coliforms involves the following:

1. Flame-sterilized the tips of blunt-ended forceps and allow to cool between successive manipulations of the filters.
2. Place a sterile absorbent pad in a sterile Petri dish.
3. Add broth medium to saturate the pad and remove the excess broth.
4. Sterilize the filter apparatus and assemble by placing a sterile filter membrane on the membrane support.
5. Mix the sample thoroughly by inverting the sample bottle several times, and put the volume to be tested into the previously sterilized filtration apparatus. The appropriate volume of sample should be selected in accordance with the type of water being tested.
6. Apply a vacuum to the filter apparatus to draw the sample through the filter membrane. Disconnect the vacuum and dismantle the apparatus.
7. Using sterile forceps, remove the membrane filter from the filter apparatus and transfer it to the nutrient pad in the Petri dish. Lower the membrane, grid side uppermost, carefully onto the nutrient pad, making sure that no air bubbles are trapped between the pad and the filter.
8. Replace the lid on the Petri dish and label with the sample identification code using a wax pencil or waterproof pen.
9. Incubate the Petri dish at ambient temperature for 2 – 4 hours to allow stressed bacteria to resuscitate.
10. Incubate the Petri dish at the selected temperature for 18 – 24 hours.
11. Following incubation, count all colonies with a morphology, typical of the bacterium and the medium used. Calculate and express the result in colony-forming units (CFU) per 100 ml of sample.

Experiment No. 04

Test for Thermotolerant (Faecal) Coliforms by Membrane Filtration Method (MFM)



Test for Thermotolerant (Faecal) Coliforms by Membrane Filtration Method (MFM)

[*Courtesy: WHO Guideline for Drinking Water Quality, 2nd Edition, Volume-3, 1997*]

Principle

In contrast to the multiple-tube method, the membrane –filtration method gives a direct count of total coliforms and thermotolerant coliforms present in a given sample of water. The method is based on the filtration of a known volume of water through a membrane filter consisting of a cellulose compound with a uniform pore diameter of 0.45 or o.2μm; the bacteria are retained on the surface of the membrane filter. When the membrane containing the bacteria is incubated in a sterile container at an appropriate temperature with a selective differential culture medium, characteristics colonies of thermotolerant coliforms develop, which can be counted directly.

Volume of Water Sample for Filtration

Since the filtration area is relatively small, it can support the growth of only a limited number of colonies: the optimum number is between 20 and 80, with a maximum of 200. If this figure is exceeded, very small typical colonies or superimposed colonies may develop, or there may be growth inhibition due to overpopulation. The choice of the volume of sample to be filtered will depend on the type of water. Examples of typical volumes are provided in Table 1.

Equipment and Glassware

In addition to the basic equipment and glassware used in the multiple-tube method, the following items are needed for the membrane –filtration technique:

- ***Membrane-filtration apparatus:*** Including an electric or hand-powered vacuum pump, a vacuum flask (e.g. an Erlenmeyer side-arm flask), and a filter support.
- ***Reusable Petri dishes:*** Made from glass or metal (disposable plastic Petri dishes may also be used).
- ***Blunt-ended forceps:*** For picking up membrane filters.
- ***Reusable (autoclavable) bottles:*** For culture media (e.g. 25 ml polypropylene bottles).
- ***A magnifying lens:*** With ×4 or ×5 magnification for examining and counting the colonies on the membrane filters.
- ***A boiling bath/pan:*** If filtration apparatus is to be disinfected in boiling water between analysis.
- ***Sterile pipettes:*** 1 ml 10 ml.
- ***A graduated cylinder:*** 100 ml.

  In addition to the consumables needed for the MPM, the following are required:

- ***Membrane filters:*** 47 – 50 mm in diameter, with a pore diameter of 0.45 μm. Singly packed, presterilized membrane filters are very convenient. Unsterilized membrane filters can also be used,

however, and should be wrapped in paper packets in convenient numbers (depending on the number of water samples to be tested). These can then be sterilized in the autoclave and dried by rapid exhaustion of the steam.

- ***Nutrient absorbent pads:*** Filter-paper discs about 1 mm thick, with the same diameter as the membrane filters.
- ***Culture media:*** Different types are available.
- ***Wax pencils:*** For labeling Petri dishes.
- ***Polythene bags:*** For wrapping Petri dishes if a dry incubator is used, to prevent drying of the sample and media.

Culture Media and Dilution Water

Various media can be used for the examination of coliform organisms by the membrane-filtration method. Of these, lactose Tergitol agar, lactose TTC Tergitol agar, and membrane lauryl sulfate lactose broth may be used for coliform organisms at 36 or 37^{0}C and for thermotolerant coliform organisms at 44^{0}C or 44.5^{0}C. Membrane faecal coliform (MFC) broth should be used only at 44 or 44.5^{0}C for thermotolerant coliform counts. Although the use of all these media for the detection of presumptive coliform organisms is based on the fermentation of lactose, the characteristic reaction varies with each medium, as shown in Table 1.

Although it is possible to prepare the media from the basic ingredients, this may be impractical in a small laboratory. The use of dehydrated media is therefore recommended. The media can be prepared as a broth and used together with nutrient absorption pads, or as solid agar plates. The broth may be solidified by the addition of 1.2 –1.5% agar before boiling.

Procedure

The procedure generally used is described here, but different types of filtration units and equipment exist.

1. Connect the Erlenmeyer (side-arm) flask to the vacuum source (turned off) and place the porous support in position. If any electric pump is used, it is advisable to put a second flask between the Erlenmeyer flask and the vacuum source; this second flask acts as a water trap, and thus protects the electric pump.
2. Open a sterile Petri dish and place a sterile absorbent pad in it.
3. Add broth medium to saturate the pad; remove excess broth.
4. Assemble the filtration unit by placing a sterile membrane filter on the porous support, using forceps sterile by flaming.
5. Place the upper container in position and secure it. (The type of clamp used will depend on the type of equipment.)

6. Pour the volume of sample chosen as optimum for the type of water into the upper container. If the test sample is less than 10 ml, at least 20 ml of sterile dilution water should be added to the top container before filtration. Apply the vacuum.
7. Take the filtration unit apart and, using the sterile forceps, place the membrane filter in the Petri dish on the pad with the grid side up. Make sure that no air bubbled are trapped between the pad and the filter.
8. Leave the Petri dish at room temperature or at 35 to 37^0C for 2 – 4 hours, for resuscitation of stressed microbes.
9. Place the dishes in an incubator at 44 $\pm$ 0.5^0C for 18 to 24 hours with 100% humidity. Alternatively, tight-fitting or sealed Petri dishes may be placed in waterproof plastic bags for incubation.
10. Submerge the bags in a water-bath maintained at 44 $\pm$ 0.5^0C for 18 to 24 hours. The plastic bags must be below the surface of the water throughout the incubation period. They can be held down by means of a suitable weight, e.g. a metal rack.

The colonies of thermotolerant coliform bacteria should be identified from their characteristics on the medium used. The number of thermotolerant coliforms per 100 ml is then given by:

$$\text{Thermotolerant coliform per 100 ml} = \frac{no..of\ .thermotolerant..coliform..colonies..counted}{no..of\ .ml.of\ .sample.filtered} \times 100$$

Table 1: Colony characteristics following analysis by the membrane-filtration method

Medium	**Colony characteristics**	
	Total coliforms at 35-37^0C	Thermotolerant coliforms at 44-45.5^0C
Lactose TTC[c] agar with Tergitol[7]	Yellow, orange or brick-red coloration with yellow central halo in the medium under the membrane	As for total coliforms at 35-37^0C
Lactose agar with Tergitol[7]	Yellow central halo in the medium under the membrane	As for total coliforms at 35-37^0C
Membrane-enriched Teepol broth	Yellow colour extending on to the membrane	As for total coliforms at 35-37^0C
Membrane lauryl sulfate broth	Yellow colour extending on to the membrane	As for total coliforms at 35-37^0C
Endo agar or broth	Dark red colour with golden-green metallic sheen	---
LES-Endo agar	Dark red colour with golden-green metallic sheen	---
Membrane faecal coliform (MFC) broth	---	Blue colonies

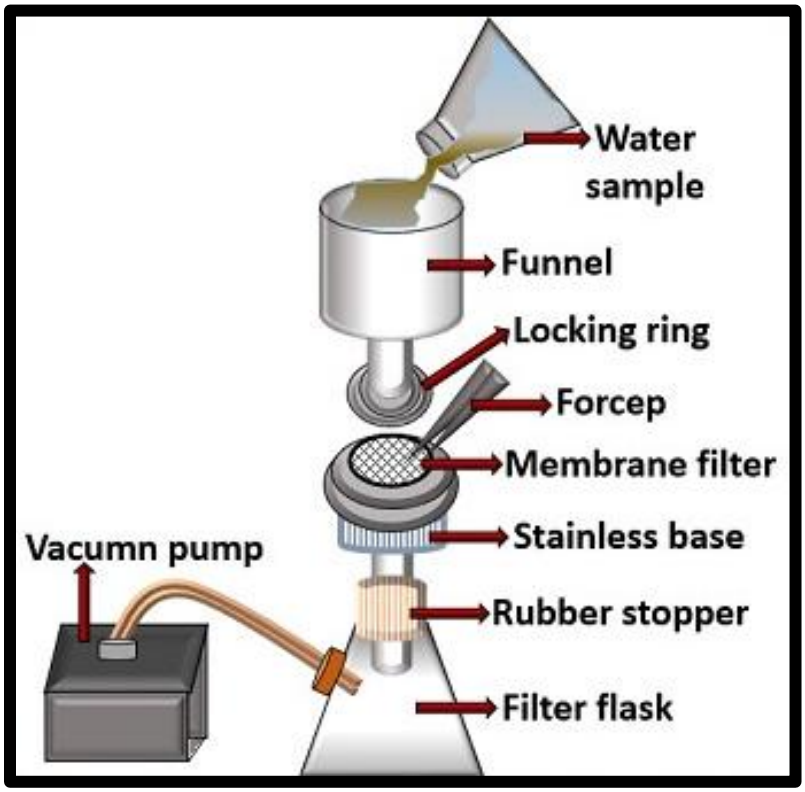
[a] Adapted from ISO 9308-1: 1990. Detection and enumeration of coliform organisms, thermotolerant coliform organisms and presumptive Eschenchia coli – Part 1. Medium filtration method.

[b] Tergitol [7] and Teepol are examples of suitable products available commercially. This information is given for the convenience of the user and does not constitute an endorsement of these products by ISO or WHO.

[c] 2.3.5-Triphenyltetrazolium chloride.

Experiment No. 04

Test for Thermotolerant (Faecal) Coliforms by Multiple tube [MPN] Method



Test for Thermotolerant (Faecal) Coliforms by Multiple tube [MPN] Method

[*Courtesy: WHO Guideline for Drinking Water Quality, 2nd Edition, Volume-3, 1997*]

In the multiple-tube method, a series of tubes containing a suitable selective broth culture medium is inoculated with test portions of a water sample. After a specified incubation time at a given temperature, each tube showing gas formation is regarded as "presumptive positive" since the gas indicates the possible presence of coliforms. However, gas may also be produced by other organisms, and so a subsequent confirmatory test is essential. The two tests are known respectively as the ***presumptive test*** and the ***confirmatory rest***.

For the confirmatory test, a more selective culture medium is inoculated with material taken from the positive tubes. After an appropriate incubation time, the tubes are examined for gas formation as before. The most probable number (MPN) of bacteria present can then be estimated from the number of tubes inoculated and the number of positive tubes obtained in the confirmatory test, using specially devised statistical tables. This technique is known as the MPN method.

1. Inoculation

Different test portions to provide tenfold serial dilution steps may be used, the dilutions being based on the anticipated number of coliform bacteria in the water sample being tested. The reliability of the result obtained depends on the number of tubes inoculated with each test portion; in certain instances, the number can be reduced to three in each dilution step. Each combination of inoculated tubes will have its own table of MPN values.

2. Unpolluted and Treated Water

Water in or entering the distribution system may generally be assumed to contain little or no pollution. In this case, it is recommended that one 50 ml plus five 10 ml volumes of water sample should be inoculated into the tubes; five tube should each contain 10 ml and one tube 50 ml of double-strength medium.

3. Polluted Water

Water suspected to be more highly contaminated, e.g. untreated water from certain raw water sources, should be examined using different inoculation volumes in tenfold dilution steps. The following inoculations are normally made:

- 10 ml of sample to each of five tubes containing 10 ml of double-strength medium;
- 1.0 ml of sample to each of five tubes containing 10 ml of single-strength medium;
- 1.0 ml of a 1:10 dilution of sample (i.e. 0.1 ml of sample) to each of five tubes containing 10 ml of single-strength medium

If the sample is expected to be highly contaminated, aliquots of 1.0 ml of tenfold serial dilution from each dilution step are inoculated into five tubes that each contains 10 ml of single-strength medium. If the workload

is very heavy and the time available is limited, the number of tube can be reduced to three in each series. Statistically, however, inoculation of five tubes with each sample volume produces a more reliable MPN result.

4. Equipment and Supplies

The following laboratory equipment and glassware are essential:

- ***Autoclave:*** Required for sterilizing the culture media. Its size should be determined by the number and type of samples to be taken. Operation of the autoclave should be strictly in accordance with the manufacturer's instructions and should ensure that all the air in the chamber is replaced by steam. Sterilization should be achieved in not more than 30 minutes. Strict adherence to recommended sterilization temperatures and times for different types of culture media is essential. Racks are needed to hold tubes and bottles of prepared culture media in the autoclave.
- ***Incubator(s) or water-baths:*** Must each fitted with a temperature control and should be capable of maintaining a uniform temperature correct to 35 or 37 $\pm$ 0.5^0C and/or 44 or 44.5 $\pm$ 0.25^0C. The choice of temperature depends on the indicator bacteria and the medium used. The temperature of incubators and water-baths fitted with temperatures placed at representative points should be monitored periodically (preferably daily). Stainless-steel racks should be fitted to hold sample tubes.
- ***Balance:*** Needed for weighing powdered culture medium. It should have an accuracy of 0.05 g. A weighing scoop is also required. (No balance is required if culture media are available in suitable preweighed quantities.)
- ***Water distillation apparatus, hose and container:*** Required to produce nontoxic water, i.e. water free from any substances that can interfere with bacterial growth. The container for the distilled water should have a volume of at least 5 liters and be fitted with a tap.
- ***Pipettes:*** 1 ml and 10 ml, with cotton plugs at the mouthpiece, are required. The 1 ml pipettes should be graduated in 0.1 ml increments and are used for preparing dilutions; the 10 ml pipettes are used for the addition of samples to tubes containing media. Any pipettes with chipped or broken tips should be discarded. Glass pipettes can be conveniently stored in a sterilizable metal container; alternatively, disposable plastic pipettes can be used. A separate container should be employed for each size of pipette. Pipettes may also be wrapped individually in paper and heat-sterilized. Pipette canisters and bulbs are also needed, as is a container for discarded pipettes.
- ***Test-tubes and racks:*** Tubes can be 20 × 150 mm in size for 10 ml sample volumes plus 10 ml of culture medium (screw caps are not recommended for fermentation media). The rack should be large enough to accommodate culture tubes of the largesse diameter used.
- ***Bottles:*** Used for the larger volumes consisting of 50 ml of sample and 50 ml of culture medium. They should have loose-fitting caps and ideally be calibrated with 50 ml and 100 ml marks.

- ***Media preparation equipment:*** Glass or stainless steel containers (usually flasks) are required. Any heating equipment and stirrers used in the preparation of media should be clean and free from soluble toxic materials.
- ***Gas burner:*** A Bunsen or similar burner is adequate.
- ***Culture tubes containing inverted vials (Durbam tubes):*** Each should be large enough for a vial, completely filled with medium, to be submerged in it.
- ***Inoculation loop and holder:*** Lengths of 24 or 26 gauge wire (7.5 – 10 cm) should be used. Nichrome wire is acceptable, but platinum-iridium is better. The wire is set in a handle made of metal or glass, of diameter similar to that of a pencil. To make the inoculation loop, the wire is bent to form a circle 3 – 4 mm in diameter.
- ***Dispenser:*** For sodium thiosulfate solution (see below).
- Cleaning and maintenance equipment: Item such as brushes for cleaning tubes, bottles, etc., a waste bin, and a tool kit are required.
- ***Safety equipment:*** There should be an adequate first-aid kit and a fire extinguisher or other means of fire control in every laboratory.
- ***General laboratory equipment:*** Various sizes of round and Erlenmeyer flask, beakers, stands, glass or unbreakable plastic measuring flasks, spatulas, etc. are required.

The following equipment is also desirable in a laboratory:

- ***Refrigerator:*** For the storage of prepared culture media.
- ***Hot air sterilizer:*** For sterilizing pipettes.

The following consumable items are required:

- ***Culture medium:*** Table 1 describe the uses for the various media; see also section 5.
- ***Laboratory disinfectant:*** For cleaning laboratory surfaces and the pipette discard bin.
- ***Detergent:*** For washing glassware, etc.
- ***Sodium thiosulfate solution:*** Required when chlorinated supplies are tested. Sodium thiosulfate neutralizes any residual chlorine in samples at the time of collection, preventing it from acting on any microorganisms present in water samples.
- ***Autoclave tape.***
- ***Diluent:*** typical diluents include Ringer's solution and phosphate-buffered saline.

Table 1: Culture media for MPN[a]

Medium	Uses	Incubator temperature	Remarks
MacConkey broth	Presumptive solution of coliform bacteria	35 ± 0.5^{0}C or 37 ± 0.5^{0}C	Traditional medium for the presumptive isolation of coliform bacteria by MPN. The quality of bile salts can vary and may affect the result.
Lauryl tryptose (lactose) broth	Presumptive solution of coliform bacteria.	35 ± 0.5^{0}C or 37 ± 0.5^{0}C	---
	Confirmation of thermotolerant coliform bacteria	44^{0}C	---
Improved formate lactose glutamate medium	Presumptive solution of coliform bacteria.	35 ± 0.5^{0}C or 37 ± 0.5^{0}C	This is a selective medium because it contains chemically defined nutrients that can be utilized only by a limited number of bacterial species. The composition of the medium is complex and specially care is required during preparation.
Brilliant green lactose (bile) broth; EC	Confirmation of coliform bacteria. Confirmation of thermotolerant coliform bacteria	35 ± 0.5^{0}C or 37 ± 0.5^{0}C	Media for gas production.
Tryptone water	Production of indole for confirmation of Escherichia coli	44^{0}C	The formation of indole detected by the addition of "Kovacs reagent" to tryptone water after incubation with gas production from lactose at 44^{0}C indicates the presence of E.Coli.

[a] Adapted from ISO 9308-2: 1990. Detection and enumeration of coliform organisms, thermotolerant coliform organisms, and presumptive Escherichia coli – Part 2: Multiple tube (most probable number) method.

[b] To make Kovacs reagent, dissolve 5 gm *p*-dimethylaminobenzaldehyde in 75 ml amyl (or isoamyl) alcohol, and add 25 ml concentrated hydrochloric acid slowly. Store at 4^{0}C in the dark.

5. Culture Media and Dilution Water

Commercially available dehydrated media simplify the preparation of culture broths and are therefore recommended for laboratory work. Various manufacturers produce these media as powers, which can then be easily weighted out, dissolved in distilled water, and dispensed into culture tubes before sterilization.

5.1 Preparation of media

Media should be prepared in accordance with the manufacturer's instructions, as follows:

(a) Dissolve the stated amount of the dehydrated medium in distilled water to obtain the double-strength or single-strength presumptive medium (for confirmatory analysis, only single-strength medium is used).

(b) Dispense the requisite volume into culture tube containing an inverted Durham tube, and cap the culture tubes.

(c) Sterilize in an autoclave or pressure cooker at 115^{0}C for 10 minutes (or in accordance with the manufacturer's specifications). It is particularly important that media containing disaccharides, e.g. lactose, are not autoclaved at higher temperatures.

(d) The sterilized medium may be stored at room temperature (Approximately 25^0C) or, ideally, at 2-8^0C. Media should in any case be warmed to room temperature before use to ensure that all components have resolved. In addition, since sever days, are light-sensitive, the solution should be protected from exposure to light.

5.2 Preparation of dilution water

A special buffered, sterilized water is used to make sample dilutions for inoculation into the culture medium. It is prepared from a concentrated stock solution of phosphate buffer. To make that stock solution, dissolve 34 gm of potassium dihydrogen phosphate (KH_2PO_4) in 500 ml of distilled water. The pH should be checked with a pH-meter; it should be 7.2. It can be increased if necessary, by adding a few drops of sodium hydroxide solution (4.0 gm dissolved in 1000 ml of distilled water). Then add sufficient distilled water to make up to a 1 liter. When the stock solution is not in use, it should be stored in a tightly closed bottle at 4 – 10^0C, to prevent microbial growth.

When using the dilution water, add 1.25 ml of stock phosphate solution to 1 liter of distilled water and dispense into bottles for sterilization in the autoclave. Before sterilization, loosen the stoppers of the bottles, Sterilize for 15 minutes at 121^0C. Tighten the stoppers after sterilization and store the dilution water in a clean place until needed.

An alternative dilution water can be prepared by the addition of magnesium chloride and has been shown to give a slightly higher recovery rate. Other alternatives include a 0.1% solution of peptone in distilled water (final pH 6.8), Ringer's solution, and physiological saline (9 gm sodium chloride per liter). These should be sterilized after dispensing into bottles, as described above.

6. Application to Unpolluted Water

6.1 Procedure

The procedure to be used for testing relatively unpolluted water, such as treated water from waterworks, is described below.

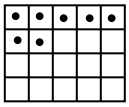
(a) Remove the cap from the sample bottle.

(b) With the stopper in position, sake the bottle vigorously to achieve a homogeneous dispersion of bacteria. (If the bottle is completely full, remove the stopper and discard 20 – 30 ml of water; then replace the stopper and shake. This ensures through mixing.)

(c) With a sterile 10 ml pipette, inoculate 10 ml of the sample into each of five tubes containing 10 ml of presumptive broth (double strength). Add 50 ml of sample to a tube containing 50 ml of presumptive broth. It is advisable to shake the tubes gently to distribute the sample uniformly throughout the medium.

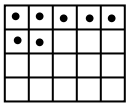
(d) Incubate the tubes at 35^0C or 37^0C for 24 hours.

(e) At the end of the 24 hours incubation period, examine each tube for the presence of gas. If present, gas can be seen in the Durham tube. If none is visible, gently shake the tube; if any effervescence (stream of tiny bubbles) is observed, the tube should be considered positive.



(f) Using a table like the one shown here, record the number of positive tubes after 24 hours.

(g) Reincubate negative tubes for a further 24 hours period. At the end of this period, check the tubes again for gas production as in (e) above. Gas production at the end of either 24- or 48-hour's incubation is presumed to be due to the presence of coliforms in the sample.

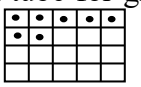


(h) Record the number of positive tubes after 48 hours.

(i) The confirmatory test should be carried out at the end of both the 24 hours and the 48 hours incubation. Using a sterile loop from each presumptive positive tube into two tubes containing respectively confirmatory broth and tryptone water. (Sterilize the inoculation loop before each transfer by flaming and allow to cool.)

(j) To confirm the presence of thermotolerant coliforms, incubate the subculture tubes from each presumptive positive tube for 24 hours at 44 ± 0.5^0C.

(k) At the end of 24 hours' incubation, examine each broth tube for growth and the presence of gas in the Durham tube. Enter the results on the table as shown.



(l) To each tube of tryptone water, add approximately 0.1 ml of Kovacs reagent (see Table 1) and mix gently. The presence of indole is indicated by a red colour in the Kovacs reagent, forming a film over the aqueous phase of the medium.

(m) Confirmatory tests positive for indole, growth, and gas production show the presence of E. coli. Growth and gas production in the absence of indole confirms thermotolerant coliforms.

Table 2: MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when one 50 ml and five 10 ml test portions are used)

No. of tubes giving a positive reaction		MPN (per 100 ml)	95% confidence limits	
1 of 50 ml	5 of 10 ml		Lower	Upper
0	0	<1	-	-
0	1	1	<1	4
0	2	2	<1	6
0	3	4	<1	11
0	4	5	1	13
0	5	7	2	17
1	0	2	<1	6
1	1	3	<1	9
1	2	6	1	15
1	3	9	2	21
1	4	16	4	40
1	5	>18	-	-

6.2 Determination of MPN

For treated water, where one 50 ml and five 10 ml portions are inoculated, the MPN can be found from the test results by means of Table 2.

7. Application of Polluted Water (Full Method)

7.1 Procedure:

The procedure to be used for the testing of water that is expressed to be polluted, even though it may have been treated, is shown below and is essentially similar to that described in section - 6, with the exception that several dilutions are used.

(a) Arrange three rows of five tubes each in a test-tube rack. The tubes in the first row (F_1) hold 10 ml of double-strength presumptive medium while the tubes in the second and third rows (F_2, F_3) contains 10 ml of single-strength presumptive medium.

(b) With a sterile pipette add 10 ml of sample to each of the five tubes in row F_1.

(c) With a sterile pipette, add 1 ml of sample to each of the five tubes in row F_2.

(d) Prepare a 1: 10 dilution of the sample by adding 1 ml of sample to 9 ml of dilution water (use a 1 ml sterile pipette). Recap the bottle containing the diluted sample and shake it vigorously.

(e) With another sterile pipette add 1 ml of the 1: 10 dilution to each of the five tubes in row F_3.

(f) After gently shaking the tubes to mix the contents, incubate the rack with the 15 tubes at 35^0C or 37^0C for 24 hours. Then proceed in the same way as for unpolluted water.

7.2 Determination of MPN

The MPN is found in a similar way to that described in section 6 but, because of the large number of tubes involved, a more complicated table – Table 3 must be used.

The following example shows how the results are obtained.

Suppose that, after confirmation of the presence of thermotolerant (faecal) coliforms, the following results are obtained:

- 5 positive tubes in row F_1 (sample volume inoculated, 10 ml)
- 3 positive tubes in row F_2 (sample volume inoculated, 1 ml)
- 1 positive tubes in row F_3 (sample volume inoculated, 0.1 ml)

Table 3: MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when five 10 ml, five 1 ml and five 0.1 ml test portions are used)

No. of tubes giving a positive reaction			MPN (per 100 ml)	95% confidence limits	
5 of 10 ml	5 of 1 ml	5 of 0.1 ml		Lower	Upper
0	0	0	<2	<1	7
0	1	0	2	<1	7
0	2	0	4	<1	11
1	0	0	2	<1	7
1	0	1	4	<1	11
1	1	0	4	<1	11
1	1	1	6	<1	15
2	0	0	5	<1	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	9	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34
3	2	1	17	5	46
3	3	0	17	5	46
4	0	0	13	3	31
4	0	1	17	5	46
4	1	0	17	5	46
4	1	1	21	7	63
4	1	2	26	9	78
4	2	0	22	7	67
4	2	1	26	9	78
4	3	0	27	9	80
4	3	1	33	11	93
4	4	0	34	12	93
5	0	0	23	7	70
5	0	1	31	11	89
5	0	2	43	15	110
5	1	0	33	11	93
5	1	1	46	16	120
5	1	2	63	21	150
5	2	0	49	17	130
5	2	1	70	23	170
5	2	2	94	28	220
5	3	0	79	25	190
5	3	1	110	31	250
5	3	2	140	37	340
5	3	3	180	44	500
5	4	0	130	35	300
5	4	1	170	43	490
5	4	2	220	57	700
5	4	3	280	90	850
5	4	4	350	120	1000
5	5	0	240	68	750
5	5	1	350	120	1000
5	5	2	540	180	1400
5	5	3	920	300	3200
5	5	4	1600	640	5800
5	5	5	>1800	-	-

The results can thus be coded as 5-3-1; represent the confirmatory test for thermotolerant coliforms. Table 3 indicates that a coded result of 5-3-1 (5 ×10 ml positive, 3 ×1 ml positive, 1× 0.1 ml positive) gives an MPN value of 110, i.e., the water sample contains an estimated 110 coliforms per 100 ml.

Next, consider an example of heavy polluted water. The procedure outlined above may give a coded result of 5-5-5. Such a result does not give a definite MPN value. When such very contamination is suspected it is usual to inoculate more than three dilutions in a series of factors of 10. This series of 10-fold dilution should be made in such a way that a negative result is likely for at least the highest dilution incubated. If 5 ×1.0 ml, 5 ×0.1 ml, 5× 0.01 ml, and 5 ×0.001 ml is initially inoculated and a confirmed coded result of 5-5-4-1 is obtained, only three of these results should then be used to obtain the MPN value from Table 5. These should be selected by choosing the smallest sample volume (in this case, 0.1 ml) for which all the tubes give a positive result, and the two next succeeding higher dilutions. The coded result of these three volumes is then used to obtain the MPN value from Table 5. In the above example, the result5-4-1 would be chosen, representing volume of 0.1,0.01, and 0.001 ml of the sample. The MPN value obtained from Table 3 should be multiplied by 100 to obtain the MPN for this particular sample (see below); in this case, the result is 17000 per 100 ml.

Sometimes the laboratory worker may find it difficult to determine the multiplying factor to be used to obtain the appropriate MPN for the sample tested. A simple way to determine the MPN is to divide the MPN value obtained from Table 5 by the sample volume represented by the middle number in the chosen code. For example, consider a chosen code of 5-2-0, in which the 2 represents a sample volume of 0.01 ml (see Table 4). From Table 3, MPN for a code of 5-2-0 is 49. The MPN value for the sample tested will therefore be: (49/0.01) = 49 ×100 = 4900.

Table 4: Example of multiplying factors for determination of the MPN for different dilutions of sample

No. of tubes giving a positive reaction						Coded result chosen	Multiplying factor for MPN
Example	5 of 1 ml	5 of 0.1 ml	5 of 0.01 ml	5 of 0.001 ml	5 of 0.0001 ml		
1	5	5	2	0	0	5-2-0	100
2	5	5	4	1	0	5-4-1	100
3	5	3	0	0	0	5-3-0	10
4	5	5	5	3	1	5-3-1	1000
5	0	1	0	0	0	0-1-0	10

Table 5: MPN values per 100 ml of sample and 95% confidence limits for various combinations of positive and negative results (when three 10 ml, three 1 ml and three 0.1 ml test portions are used)

No. of tubes giving a positive reaction			MPN (per 100 ml)	95% confidence limits	
3 of 10 ml	3 of 1 ml	3 of 0.1 ml		Lower	Upper
0	0	1	3	<1	9
0	1	0	3	<1	13
0	0	0	4	<1	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	49
2	2	0	21	4	47
2	2	1	28	10	149
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	379
3	1	0	48	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	1	460	71	2400
3	3	2	1100	150	4800

Examples are given in Table 4 of the factors to be used to multiply the MPN value found in order to obtain the appropriate MPN for different dilutions.

8. Application to Polluted Water: "Shorter Method"

The procedure for the shorter method is almost identical to that described in section 7, with the single difference that only three tubes of each sample volume are inoculated, instead of five. This requires the use of a different table – Table 5 for determining the MPN.

9. Direct Thermotolerant Coliform Method

If *unchlorinated* water from small community water supplies is tested and only the number of thermotolerant coliforms is of interest, a direct multiple-tube method can be used. This is recommended for use where the total coliform result is not of great significance, e.g., in small-community supplies in developing countries or where space, time or facilities are limited. The method is based on the normal MPN procedure, but the tubes are incubated directly in a water-bath at 44.5 $\pm 0.2^0$C, without previously incubating at 35 or 37^0C for 24 hours and testing for total coliforms.

The procedure is similar to that described for the examination of polluted water, except that MacConkey broth is used as the presumptive medium. Prepare 15 tubes of sample and medium, as described and then proceed as shown below.

(a) After gently shaking the tubes to mix the contents, incubate the 15 tubes at 44^0C for 24 hours.

(b) Observe each tube for the presence of gas and enter the number of positive tubes after 24 hours in the appropriate table.

(c) Negative tubes should be reincubated for a further 24-hour period, after which they should be observed for the presence of gas.

(d) Confirm the presumptive results after 24 and 48 hours by transferring a loopful of broth to a confirmatory broth and incubating at 44^0C for 24 hours.

(e) The presence of thermotolerant coliforms is confirmed if gas is present in the confirmatory broth after 24 hours at 44^0C. Determine the MPN from Table 3 as before.

10. Selection of Tubes for Confirmatory Test

Any bacteriological analysis should always include the confirmatory test. If only five 10 ml portions are tested, the confirmatory test for coliforms and thermotolerant coliforms must be carried out on all tubes showing gas production. However, if the inoculation involved five (or three) tubes for each of, or more than, three sample volumes (e.g. 10,1.0,0.1,0.01, and 0.001 ml), it is not necessary to carry out confirmatory tests on all the positive tubes.

If all five (or three) tubes of two or more consecutive dilutions are positive, the set of tube should be selected that presents the smallest sample volume for which all the tubes are positive. The confirmatory test should be carried out on all these tubes and on all the positive tubes corresponding to subsequent and lower volumes. The following example should help to illustrate this procedure. After 24 hours' incubation, five tubes with 10 ml, five with 1.0 ml, five with 0.1 ml, four with 0.01 ml and one with 0.001 ml gave positive results. Thus, the confirmatory test should be carried out on the positive tubes initially inoculated with 0.1, 0.01 and 0.001 ml of sample.

11. Record Forms

The analysis of a given sample will provide several results. The form drawn up for recording these results, although it should not be complicated, must be completed. The completed form should contain the data on the sampling, which will also serve to identify the samples, those entered on the sample dispatch form, and data on the bacteriological analysis itself. A suggested comprehensive form is shown in Figure 1. Once the analysis is completed, the laboratory carried out the work should record the results obtained in a standardized form (protocol); this should follow the recommendations given. The protocol can be a very simple report, which records the sample identification information together with the result of the analysis and the appropriate classification of the water. An example of such a protocol is shown in Figure 2.

Figure 2: Suggested protocol for results of bacteriological analysis

WATER QUALITY CONTROL PROGRAMME

Bacteriological Water Analysis

Authority: …….

Community: …….

Sample No.: …….

Sample Site: …….

Place: ……

Source: …….

Sender: ……

Date of sampling ___/___/___ Time: …….

Date of analysis Time: …….

Residual free chlorine [ ] mg/liter

Results:

Total coliforms [ ] /100 ml

Faecal coliforms [ ] /100 ml

Water Bacteriology: Good / Bad

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Laboratory Technician Chief (Signed)