MICROBIOLOGY

SEMESTER IV

PAPER CODE- MBIO CC409

PAPER – ENVIRONMENTAL MICROBIOLOGY

TOPIC- Treatment and safety of drinking water (potable) water, methods to detect potability of water samples

BY:

PREETI SWARUPA
Preetipandey1920@gmail.com
Assistant Professor
Department of Microbiology
Patna Women's College

Potable water

Potable water, also called drinking or tap water, is used for sanitary purposes such as drinking, fountains, showers, toilets, hand-wash basins, cooking, etc.

According to the World Health Organization's 2017 report, *safe* drinking-water is water that "does not represent any significant risk to health over a lifetime of consumption, including different sensitivities that may occur between life stages".

More than 1.5 billion people in developing nations are still without safe drinking water. Waterborne diseases such as typhoid, cholera, dysentery, amebiasis, salmonellosis, shigellosis, and hepatitis A are still estimated to be responsible for the deaths of more than 30,000 people daily.

Parameters of potable/drinking water

Parameters for drinking water quality typically fall within three categories:

- physical
- chemical
- microbiological

Physical and chemical parameters include heavy metals, trace organic compounds, total suspended solids (TSS), and turbidity. Physical parameters affect the aesthetics and taste of the drinking water and may complicate the removal of microbial pathogens.

Chemical parameters tend to pose more of a chronic health risk through buildup of heavy metals although some components like nitrates/nitrites and arsenic can have a more immediate impact.

There is increasing concern over the health effects of engineered nanoparticles (ENPs) released into the natural environment. One potential indirect exposure route is through the consumption of contaminated drinking waters.

Microbiological parameters include Coliform bacteria, *E. coli*, and specific pathogenic species of bacteria (such as cholera-causing *Vibrio cholerae*), viruses, and protozoan parasites. Originally, fecal contamination was determined with the presence of coliform bacteria, a convenient marker for a class of harmful fecal pathogens. The presence of fecal coliforms (like *E. Coli*) serves as an indication of contamination by sewage. Additional contaminants include protozoan oocysts such as *Cryptosporidium sp.*, *Giardia lamblia*, *Legionella*, and viruses (enteric). Microbial pathogenic parameters are typically of greatest concern because of their immediate health risk.

Throughout most of the world, the most common contamination of raw water sources is from human sewage in particular human faecal pathogens and parasites.

Waterborne pathogens

The greatest microbial threat to drinking water supplies arises from the likelihood of contamination from faeces of human and animal origin containing harmful micro-organisms. Table below shows the types of waterborne pathogens that may originate in the faeces of humans or other animals; these include bacteria, viruses and protozoa and helminths (i.e. parasitic worms).

Table 2.1. ***Characteristics of waterborne pathogens***

Size (μm)	Pathogen	Resistance [1] to Chlorine	Relative [2] Infectivity
Bacteria			
0.1 - 10	*Salmonella* spp.	*Low*	*Moderate*
	Shigella spp	*Low*	*High*
	Yersinia enterocolitica	*Low*	*Low*
	Campylobacter spp.	*Low*	*Moderate*
	Escherichia coli (pathogenic)	*Low*	*Low*
	Verocytotoxigenic *E-coli including E-coli-O157*	*Moderate*	*High*
	Pseudomonas aeruginosa	*High*	*Low*
	Mycobacterium spp.		*Low*
Viruses			
0.05 - 0.1	*Rotavirus*	*Moderate*	*High*
	Astrovirus	*Moderate*	*High*
	Norovirus	*Moderate*	*High*
	Parvovirus	*Moderate*	*High*
	Adenovirus	*Moderate*	*High*
Protozoa			
4 - 15	*Entamoeba histolytica*	High	High
	Cryptosporidium spp.	High	High
	Giardia spp.	High	High
Helminths (Parasitic Worms)			
Visible	*Drancunculus medinesis*	*Moderate*	High
	Schistosoma	*Moderate*	High

Disinfection of drinking water

Killing, removal, or deactivation of harmful microorganisms can be referred to as disinfection. Destruction or deactivation of pathogenic microorganisms results in stopping their reproduction and growth. People may fall ill by consuming the contaminated water containing the pathogenic microorganisms. Disinfection and sterilization are interrelated processes, but sterilization kills all the harmful and harmless microorganisms. Hence, disinfection is a more appropriate process.

Disinfection kills or inactivates disease-causing organisms in a water supply and must provide a 99.9 percent inactivation of *Giardia lamblia* cysts and enteric viruses to protect health and to comply with the U.S. Environmental Protection Agency (EPA) regulations.

There are two kinds of disinfection:

- Primary disinfection achieves the desired level of microorganism kill or inactivation, while
- Secondary disinfection maintains a disinfectant residual in the finished water that prevents the regrowth of microorganisms.

Mechanism of disinfection

Disinfection commonly takes place because of cell wall corrosion in the cells of microorganisms, or changes in cell permeability, protoplasm or enzyme activity (because of a structural change in enzymes).

These disturbances in cell activity cause microorganisms to no longer be able to multiply. This will cause the microorganisms to die out.

Oxidizing disinfectants also demolish organic matter in the water, causing a lack of nutrients. Chemical inactivation of microbiological contamination in natural or untreated water is usually one of the final steps to reduce pathogenic microorganisms in drinking water. Combinations of water purification steps (Oxidation, Coagulation, settling, disinfection, filtration) cause (drinking) water to be safe after production.

Methods of disinfection of potable/drinking water

Methods of disinfection falls under two categories-

- Physical
- Chemical

Chemical disinfection is done through-

- Chlorine (Cl_2)
- Chlorine dioxide (ClO_2)
- Hypo chlorite (OCl-)
- Ozone (O_3)

- Halogens: bromine (Br_2), iodine (I)
- Bromine chloride (BrCl)
- Metals: copper (Cu^{2+}), silver (Ag^{+})
- Potassium Permanganate ($KMnO_4$)
- Phenols
- Alcohols
- Hydrogen peroxide
- Several acids and bases

Physical disinfection-

- Ultraviolet light (UV)
- Electronic radiation
- Gamma rays
- Heat

Chlorine

- Chlorine gas is highly oxidizing, toxic, corrosive and hazardous yellow-green gas (supplied as liquid chlorine in bullets)
- Effective against all types of microbes as both primary and secondary disinfectant
- Leaves combined and free residual chlorine in the treated water and this can be responsible for secondary disinfection
- Chlorine forms trihalomethane with organic compound present in water which is carcinogenic

Chlorine dioxide

- As a strong oxidant, ClO_2 is similar to ozone.
- It does not form trihalomethanes.
- ClO_2 is particularly effective in destroying phenolic compounds that often cause severe taste and odour problems when reacted with chlorine.
- Similar to the use of chlorine, it produces measurable residual disinfectants.
- ClO_2 is a gas and its contact with light causes it to photooxidize.
- Thus, it must be generated on-site.
- Although its principal application has been in wastewater disinfection, chlorine dioxide has been used in potable water treatment for oxidizing manganese and iron and for the removal of taste and odor.
- Its probable conversion to chlorate, a substance toxic to humans, makes its use for potable water treatment questionable.

Hypochlorite

- In households, hypochlorite is used frequently for the purification and disinfection of water
- Sodium hypochlorite and calcium hypochlorite are used in disinfection of water.
- Sodium hypochlorite (NaOCl) is a compound that can be effectively used for water purification.
- It is used on a large scale for surface purification, bleaching, odor removal and water disinfection.
- As a bleaching agent for domestic use it usually contains 5% sodium hypochlorite.
- Due to the presence of caustic soda in sodium hypo chlorite, the pH of the water is increased. When sodium hypo chlorite dissolves in water, two substances form, which play a role in for oxidation and disinfection. These are hypochlorous acid (HOCl) and the less active hypochlorite ion (OCl^-). The pH of the water determines how much hypochlorous acid is formed. While sodium hypochlorite is used, hydrochloric acid (HCl) is used to lower the pH.
- Hypochlorous acid is divided into hydrochloric acid (HCl) and oxygen (O). The oxygen atom is a very strong oxidator.

- Sodium hypochlorite is effective against bacteria, viruses and fungi. Sodium hypochlorite disinfects the same way as chlorine does.
- Advantages: It can easily and be stored and transported when it is produced on-site. Dosage is simple. Transport and storage of sodium hypochlorite are safe. Sodium hypochlorite is as effective as chlorine gas for disinfection. Sodium hypochlorite produces residual disinfectant.
- *Disadvantages*- Sodium hypochlorite is a dangerous and corrosive substance. While working with sodium hypochlorite, safety measures have to be taken to protect workers and the environment. Sodium hypochlorite should not come in contact with air, because that will cause it to disintegrate.

Ozone

- Ozone gas is unstable allotropic form of oxygen, with each of its molecules containing three oxygen items.
- It can be produced by passing high tension electric current through the stream of air in a closed chamber. The nascent oxygen so produced as by product during ozone formation is powerful oxidizing agent and removes the organic matter and bacteria from water.
- Advantages: Ozone being unstable nothing remains in water by the time it reaches in water to distribution system.
- Ozone removes the colour, taste and odour from water in addition to removing bacteria from it. The ozonized water becomes tasty and pleasant unlike the chlorinated water which becomes bitter to tongue.
- Disadvantages: It is much costlier than chlorination. It needs electricity for its manufacture and hence can be used when electricity is available easily and cheaply

No residuals can be maintained because it is highly unstable and hence it does not ensure safety against possible contamination.

Halogens: bromine (Br_2), iodine (I)

- These disinfectants are now a days are also available in form of pills and are thus very handy.
- They are not used for treating any large scale public supplies but may be used for treating small water supplies for army troops, private plants, swimming pools etc.

Bromine Chloride

- BrCl is an effective disinfectant which is economically competitive and less toxic than chlorine.
- Furthermore, it can be handled safely and can be adapted to existing chlorination systems with only minor modifications

Potassium Permanganate ($KMnO_4$)

- This is used as popular disinfectant for disinfecting well water in villages which are generally contaminated with lesser amount of bacteria.
- Advantages- Besides killing bacteria it also helps in oxidizing the taste producing organic matter hence it is added in small doses to chlorinated water also.
- It has also been used as an algaecide and for removing colour and iron from water.
- Disadvantages- Potassium permanganate though cheap, handy and useful yet can't guarantee 100% removal of bacteria.
- It can possibly remove organisms causing cholera, but is of little use against other disease organisms.
- Water treated with potassium permanganate with the passage of time, produces a dark brown precipitate, which is noticeable as a coating on porcelain vessels and is difficult to remove without scouring.

Metals: copper (Cu^{2+}), silver (Ag^{+})

- In this method of disinfection metallic silver ions and copper ions are introduced into the water by passing it through a tube containing solid silver/copper electrodes which are connected to a DC supply of about 1.5 volts.
- Electrically charged copper ions (Cu^{2+}) in the water search for particles of opposite polarity, such as bacteria, viruses and fungi. Positively charged copper ions form electrostatic compounds with negatively charged cell walls of microorganisms. These compounds disturb cell wall permeability and cause nutrient uptake to fail.
- Copper ions penetrate the cell wall and as a result they will create an entrance for silver ions (Ag^{+}). These penetrate the core of the microorganism.
- Silver ions bond to various parts of the cell, such as the DNA and RNA, cellular proteins and respiratory enzymes, causing all life support systems in the cell to be

immobilized. As a result, there is no more cellular growth or cell division, causing bacteria to no longer multiply and eventually die out. The ions remain active until they are absorbed by a microorganism.

- Advantages- The silver treatment neither imparts any taste and odour to water nor it produces any harmful effects on human body. The method removes algae and its germicidal property is retained for a considerable time, thus allowing some safeguard against future contamination
- Disadvantages- Use of silver/copper is very costly and hence not adopted for treating for public supplies. Suspended organic matter and hydrogen sulphide should be removed before using this disinfectant hence it adds to cost of disinfection

Hydrogen peroxide

- The hydrogen peroxide molecule contains one extra oxygen atom, compared to the more stable water molecule.
- The bond between the two oxygen atoms, the so-called peroxide bond, is broken while two H-O radicals are formed. These radicals quickly react with other substances, while new radicals are formed and a chain reaction takes place.
- *Advantages*- Contrary to other chemical substances, hydrogen peroxide does not produce residues or gasses. Safety depends on the applied concentration, because hydrogen peroxide is completely water soluble.
- *Disadvantages* - Hydrogen peroxide is a powerful oxidizer. It reacts with a variety of substances. It is therefore diluted during transport, as a safety measure. However, for hydrogen peroxide disinfection, high concentrations are required.
  Hydrogen peroxide slowly decomposes into water and oxygen. An elevation of temperature and the presence of pollutions enhance this process.

  The concentration of hydrogen peroxide in a solution slowly decreases. This is caused by the following reaction:
  $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$
  This is a redox reaction. Hydrogen molecules partly function as reductors and partly as oxidizers.

Alcohols

- The most feasible explanation for the antimicrobial action of alcohol is denaturation of proteins.
- The bactericidal activity of ethyl alcohol (ethanol) is found against a variety of microorganisms e.g. *Pseudomonas aeruginosa, Serratia marcescens, E, coli, Salmonella typhosa Staphylococcus aureus* and *Streptococcus pyogenes*
- Ethyl alcohol, at concentrations of 60%–80%, is a potent virucidal agent inactivating all of the lipophilic viruses (e.g., herpes, vaccinia, and influenza virus) and many hydrophilic viruses (e.g., adenovirus, enterovirus, rhinovirus, and rotaviruses but not hepatitis A virus (HAV) or poliovirus).

- Isopropyl alcohol is fully active against the lipid viruses. Studies also have demonstrated the ability of ethyl and isopropyl alcohol to inactivate the hepatitis B virus (HBV) and the herpes virus, and ethyl alcohol to inactivate human immunodeficiency virus (HIV), rotavirus, echovirus, and astrovirus.

Phenols

- In high concentrations, phenol acts as a gross protoplasmic poison, penetrating and disrupting the cell wall and precipitating the cell proteins.
- Low concentrations of phenol and higher molecular-weight phenol derivatives cause bacterial death by inactivation of essential enzyme systems and leakage of essential metabolites from the cell wall.

Several acids and bases

- Acids, bases and salts are commonly used for pH adjustment, flocculation, sedimentation, disinfection, corrosion control, water softening and taste, color and odour control.
- When an acid, base or salt is added to water, the compound will split into two separate parts. When this occurs the compound is said to have **disassociated**.
- An **acid** is a compound, which will release **hydrogen ions** (H^+) when it disassociates. The stronger the acid the more hydrogen ions the compound will release.
  An example of an acid hydrochloric acid:

  $HCl \sim\sim H^+ + Cl^-$

  Hydrorochloric Acid ~~ Hydrogen ion + Chloride ion
- When a **base** is added to water and disassociates it will release **hydroxyl ions** (OH). The stronger the base the more hydroxyl ions will be released. This is illustrated in the equation below.

  $NaOH \sim\sim Na^+ + OH^-$

  Sodium Chloride ~~ Sodium ion + Chloride ion

  A salt will not release hydrogen or hydroxyl ions when it dissociates. A good example is common salt, sodium chloride;

  $NaCl \sim\sim Na^+ + Cl^-$

  Sodium Chloride ~~ Sodium ion + Chloride ion
- Sodium hydroxide, Potassium hydroxide, Calcium hydroxide, Magnesium hydroxide, Sulfurous acid, Sulfur dioxide etc have disinfectant property.

UV rays

- Ultraviolet rays are invisible rays having wavelength 1000- 4000 mμ and can be produced by passing electric current through mercury enclosed in quartz bulbs.
- The water to be treated with ultraviolet rays should, however, be less turbid and low in colour.
- Normally it should be colourless and turbidity should not exceed 15 mg/l
- The depth of water over the bulbs should not generally exceed 10cm or so because these rays can effectively penetrate through this much distance only.
- Advantages- Sterilization with UV rays does not impart any addition taste or odour to water, as no chemicals are added. The method possesses ample scope for treating small quantities of water in hospitals and dispensaries for surgical uses or for drinking purposes for the place where cost is minor factor.
- Disadvantages- Method is very costly, needs technical knowhow, and possesses possibilities of interruption due to failure of electricity.

Gamma rays

- Gamma rays are emitted from radioisotopes, such as cobalt-60, which, because of their penetrating power, have been used to disinfect water and wastewater.

Electronic radiation

- The electron beam uses an electron generator. A beam of these electrons is then directed into a flowing water or wastewater to be disinfected.
- For the method to be effective, the liquid must flow in thin layers.

Heat

- Boiling is one heat method.
- It is highly efficacious, killing human pathogens even in turbid water and at high altitude.
- However, boiling involves the high-cost use of carbon-based fuel sources and does not provide any residual protection.
- Enteric bacteria, protozoa and viruses in liquids are sensitive to inactivation at temperatures below 100°C. Thermal inactivation has been examined in water, sewage, milk and other liquids at temperatures close to those used for pasteurization (e.g. 63 °C for 30 minutes, 72 °C for 15 seconds) and in hot water (about 60 °C).
- Only a few studies have examined thermal inactivation in liquids at temperatures approaching 100°C.
- Bacteria are particularly sensitive to heat.
- Viruses are inactivated at temperatures between 60°C and 65°C, but more slowly than bacteria.

- *Cryptosporidium parvum* oocysts are inactivated in less than 1 minute once temperatures exceed 70 °C. *Giardia* cysts gets inactivation at temperatures ranging from 50 °C to 70 °C.

Faecal Indicators in Drinking Water Control

There are hundreds of different enteric microorganisms that are known to infect humans. Enteric microorganisms are excreted in the faeces of infected individuals or animals, and may directly or indirectly contaminate water intended for human consumption

However, many waterborne pathogens are still difficult to detect and/or quantify in waters and for most of the newly recognized agents, easy methods to detect them in water samples have still to be developed. However, the routine application of these methods for the analysis of pathogens is not a reality yet and is restricted to research studies or to cases of suspected outbreaks. Therefore, the most useful tool to determine the potential presence of pathogenic microorganisms in waters is the analysis of several microorganisms classed as either "indicator, or, index" organisms.

To avoid the ambiguity in the term "microbial indicator", the following three groups are now recognized: process microbial indicators, faecal indicators and index and model organisms. Process indicators comprise a group of organisms that demonstrate the efficacy of a process; faecal indicators are those organisms that indicate the presence of faecal contamination, hence, they only infer that pathogens may be present; index and model organisms include a group or species indicative of pathogenic presence and behaviour, respectively. The most widely used indicators are coliforms (total coliforms), faecal or thermotolerant coliforms, *Escherichia coli*, enterococci (faecal streptococci or intestinal enterococci) and bacteriophages.

Coliforms

Coliform bacteria are facultative anaerobes, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with acid production in 24 to 48 h at 36 ºC, and indole-negative. Coliforms belong to the family *Enterobacteriaceae* and include *Escherichia, Enterobacter, Klebsiella* and *Citrobacter, Kluyvera, Leclercia* genera, and some members of the genus *Serratia*. These bacteria were classically used as indicators of faecal contamination of waters because they were considered to be inhabitants of the intestinal tracts of homeothermic animals. However, the ability of some coliforms to grow in natural waters, the lack of correlation between the number of coliforms and those of pathogenic microorganisms, and the detection of atypical strains has led them to become unsuitable faecal indicators. The coliform bacteria, traditionally termed the "total coliform" group, have been the primary standard for potable water in most of the world. However, many regulatory agencies have questioned its utility as an indicator. The presence of coliforms in the distribution system, while possibly due to inadequate treatment, could also be due to cross-connections or failure to maintain an adequate disinfectant residual.

Faecal Coliforms

These bacteria conform to all the criteria used to define total coliforms plus the requirement that they grow and ferment lactose with the production of acid at 44.5 ºC. For this reason, "thermotolerant coliform" would be the scientifically accurate term for this group. Bacteria in this coliform subgroup have been found to have a positive correlation with faecal contamination of warm-blooded animals. However, some thermotolerant coliform bacteria that conform to this definition also belong to the genus *Klebsiella* and have been isolated from environmental samples in the apparent absence of faecal pollution. Faecal coliforms display a survival pattern similar to these of bacterial pathogens but their usefulness as indicators of protozoan and viral contamination is limited, therefore, tended to be replaced by *E. coli* in several legislations.

Escherichia coli

E. coli is a member of faecal coliform group, being a more specific indicator for the presence of faecal contamination. In addition, *E. coli* conforms to taxonomic as well as functional identification criteria and is enzymatically distinguished by the lack of urease and presence of β-glucuronidase. One disadvantage associated with this organism as an indicator is that it has been consistently found in pristine tropical rain forest aquatic and plant systems as well as soils. Additionally, it seems to survive for short periods in aquatic temperate environments. *E. coli* is the faecal indicator of choice used in WHO Guidelines for Drinking-water Quality, and several countries include this organism in their regulations as the primary indicator of faecal pollution (*i.e.*, Europe, USA). Although it has long been known that *E. coli* can cause disease in humans, the bacteria naturally occurs in the lower part of the gut of warm-blooded animals.

Faecal Streptococci, Enterococci or Intestinal Enterococci

This group of microorganisms has received widespread acceptance as useful indicators of microbiological water quality, since: (i) they show a high and close relationship with health hazards associated with the water use, mainly for gastrointestinal symptoms; (ii) they are always present in faeces of warm-blooded animals; (iii) they unable to multiply in sewage-contaminated waters; and (iv) their die-off is less rapid than those of coliforms in water, and persistence patterns are similar to those of potential waterborne pathogenic bacteria.

Faecal streptococci, enterococci and intestinal enterococci are three synonyms used to refer to species described as members of the genus *Enterococcus*, *Enterococcus faecalis* and *E. faecium,* are the predominant species in human faeces and sewage.

Despite the definitions provided above for the indicators (total coliforms, faecal coliforms, *E. coli* and enterococci) in practical terms these are determined on the basis of the biochemical reactions evaluated in culture media that are recognized either by the appearance of characteristic colonies (with a specific colour as response to this reaction in chromogenic substrates) and/or by the emission of fluorescence.

Bacteriophages

Several bacteriophage groups have also been classically used as faecal and viral indicators, and as models to evaluate the efficiency of the chlorination of drinking waters. The proposed groups are somatic coliphages, F (male)-specific RNA bacteriophages (FRNA phages) and phages of *Bacteroides fragilis*.

Somatic coliphages are specific viruses of *E. coli* and have been commonly used as indicators of faecal and/or sewage pollution in several water types and as biotracers to identify pollution sources in surface waters and aquifers. In addition, they may also serve as indicators for assessing the removal efficiency during the treatment of water and wastewater treatment plants. On the basis of the differences in origin and ecology between enteric viruses and somatic coliphages, it is doubtful to conclude that this phage group could successfully be used in all situations as enteric viruses indicators, and they may not be a useful indicator of a distribution system integrity problem, even when the problem involves the introduction of faecal contamination.

The use of FRNA phages was proposed as faecal pollution indicators and as model viruses in water hygiene on the basis of: (i) their similar sizes and shapes to human enteric viruses; (ii) their correlation with the sewage contamination degree; and (iii) their inability to replicate in the water ecosystem. However, the low incidence of this phage group in human faeces and its low specificity for its bacterial host, suggest that they would multiply in the sewerage system. Hence, the presence of FRNA phages in water should be primarily used as an index of sewage pollution rather than faecal pollution.

Bacteroides fragilis is a strict anaerobe found in high concentrations in the human intestinal tract and dies rapidly when discharged into environmental waters. A phage of the strain HSP 40 of *B. fragilis* has been proposed as a specific index of human faecal pollution of waters, because: (i) phages against this bacterial strain are human specific and are not isolated from the faeces of other homoeothermic animals; (ii) *B. fragilis* HSP 40 phages are consistently isolated from sewage, faecally-polluted waters, and their sediments but not from unpolluted samples; (iii) the levels of phages are related to the degree of pollution; (iv) *B. fragilis* phages always outnumber human enteric viruses; and (iv) in model experiments, no replication of these phages has been observed under simulated environmental conditions. The low prevalence of these phages in waters with low and moderate levels of faecal pollution and the complex methodology for their recovery are the main drawbacks for the general use of these viruses as an indicator group

Methods to detect potability of water samples

The principal methods used in the isolation of indicator organisms from water are the membrane-filtration (MF) method, the multiple-tube (MT) or most probable number (MPN) method and presence–absence tests.

Membrane-filtration method

In the membrane-filtration (MF) method, a minimum volume of 10 ml of the sample (or dilution of the sample) is introduced aseptically into a sterile or properly disinfected filtration assembly containing a sterile membrane filter (nominal pore size 0.2 or 0.45μm). A vacuum is applied and the sample is drawn through the membrane filter. All indicator organisms are retained on or within the filter, which is then transferred to a suitable selective culture medium in a Petri dish. Following a period of resuscitation, during which the bacteria become acclimatized to the new conditions, the Petri dish is transferred to an incubator at the appropriate selective temperature where it is incubated for a suitable time to allow the replication of the indicator organisms. Visually identifiable colonies are formed and counted, and the results are expressed in numbers of "colony-forming units" (CFU) per 100 ml of original sample. This technique is inappropriate for waters with a level of turbidity that would cause the filter to become blocked before an adequate volume of water had passed through. When it is necessary to process low sample volumes (less than 10 ml), an adequate volume of sterile diluent must be used to disperse the sample before filtration and ensure that it passes evenly across the entire surface of the membrane filter. Membrane filters may be expensive in some countries.

Multiple-tube method

The multiple-tube method is also referred to as the most probable number (MPN) method because—unlike the MF method—it is based on an indirect assessment of microbial density in the water sample by reference to statistical tables to determine the most probable number of microorganisms present in the original sample. It is essential for highly turbid samples that cannot be analysed by membrane filtration. The technique is used extensively for drinking-water analysis, but it is time-consuming to perform and requires more equipment, glassware, and consumables than membrane filtration. However, the multiple-tube method may be more sensitive than membrane filtration.

The multiple-tube method depends on the separate analysis of a number of volumes of the same sample. Each volume is mixed with culture medium and incubated. The concentration of microorganisms in the original sample can then be estimated from the pattern of positive results (the number of tubes showing growth in each volume series) by means of statistical tables that give the "most probable number" per 100 ml of the original sample. The combination of sample volumes for processing is selected according to the type of water sample or known degree of contamination. Appropriate volumes of water are added aseptically to tubes or other vessels containing sterile nutrient medium of a concentration that will ensure the mixture corresponds to single-strength medium. The tube must also contain a small inverted glass tube (Durham tube) to facilitate the detection of gas production. Growth in the medium is confirmed by visible turbidity and/or a colour change. Tubes are incubated without resuscitation, and the number of positive reactions is recorded after 24 and/or 48 hours, depending on the type of analysis.

Most Probable Number Method (MPN) MPN test is performed in 3 steps-

- Presumptive test
- Confirmatory test
- Completed test

Presumptive test:

The presumptive test is a screening test to sample water for the presence of coliform organisms. If the presumptive test is negative, no further testing is performed, and the water source is considered microbiologically safe. But if test is found positive further test is required i.e. confirmatory test.

Preparation of the Medium- Prepare medium (either Mac Conkey broth or Lactose broth). Dispense the medium in 10 tubes and add an Durham's tube in inverted position. Examine the tubes to make sure that the inner vial is full of liquid with no air bubbles. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then inoculate the tubes with polluted water.

Confirmed/confirmatory test:

Some microorganisms other than coliforms also produce acid and gas from lactose fermentation. In order to confirm the presence of coliform, confirmatory test is done. • From each of the fermentation tubes with positive results (presumptive test) transfer one loopful of medium to 3 ml brilliant green lactose broth (BGLB) fermentation tube, to an agar slant and 3 ml tryptone water. Incubate the inoculated BGLB fermentation tubes at 37°C and inspect gas formation after 24 ± 2 hours. If no gas production is seen, further incubate up to maximum of 48 ±3 hours to check gas production. The agar slants should be incubated at 37°C for 24± 2 hours and Gram-stained preparations made from the slants should be examined microscopically.

The absence of gas formation in BGLB broth or the failure to demonstrate Gram-negative, non-spore- forming bacilli in the corresponding agar slant constitutes a negative test (absence of coliforms in the tested sample).

The formation of gas in BGLB broth and the demonstration of Gram negative, non-spore-forming bacilli in the corresponding agar indicate the presence of a member of the coliform group in the sample examined. Further test is done i.e. completed test

Completed test:

Since some of the positive results from the confirmatory test may be false, it is desirable to do completed tests. For this inoculum from each positive tube of the confirmatory test is streaked on a plate of EMB or Endo agar. In this process, a loopful of sample from each positive BGLB tubes is streaked onto selective medium like Eosin Methylene Blue agar or Endo's medium. One plate each is incubated at 37°C and another at 44.5± 0.2°C for 24 hours.

Presence–absence tests

Presence–absence tests may be appropriate for monitoring good-quality drinking water where positive results are known to be rare. They are not quantitative and, as their name suggests, they indicate only the presence or absence of the indicator organism. Such results are of very little use in countries or situations where contamination is common; Thus, presence–absence tests are not recommended for use in the analysis of surface waters, untreated small-community supplies, or larger water supplies that may experience occasional operational and maintenance difficulties.