Lab - 9

Microbial Examination

Two types of microbiological tests for water samples will be described in the sections that follow. First, the multiple tube fermentation (MPN) test, which is a measure of the most probable number (statistically) of organisms that are present in the water sample. Second the plate-count procedure, which gives an actual count of the number of bacteria in water.

None of the methods described in the following section actually measure the number of pathogenic organisms in water. They actually measure the concentration of coliform, i.e., "indicator" organisms, the presence of which in high concentrations would indicate the possible presence of pathogenic organisms.

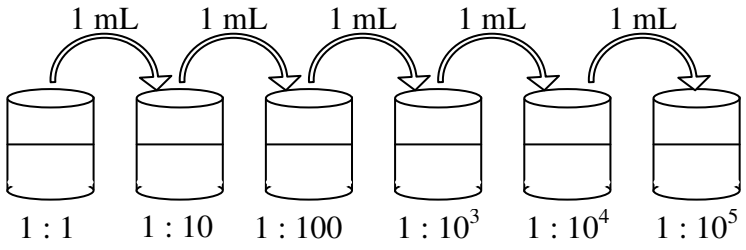
Certain precautions must be taken for both tests to ensure correct results and avoid contamination of the test from outside sources.

Precautions:

- It is absolutely necessary to maintain a sterile environment while conducting microbiological tests. The objective is to ensure that the **only** source of microbes is the contaminated water, i.e., microbes, which were not originally present in the water sample, are excluded from experimental procedure. This is easier said than done, since microbes are everywhere, on our hands, in the atmosphere, on almost all containers and apparatus normally used in the experimental procedure, and even in the nutrient broth used for incubation and colony formation.
- The glassware, pipettes, dilution water, petri dishes, test tubes and nutrient broth used for microbiological experiments must be decontaminated beforehand. This is done by sterilization, or by UV disinfection.
- The actual experiments are conducted under a laminar hood, which is sanitized beforehand by UV light. An air filter or constant flow of air is maintained from the hood to outside, so that microbes may not enter the hood during experiments.

Serial Dilution Technique:

- Often the bacterial contamination of waters is high enough to require dilution before enumeration by standard techniques.
- Water samples are diluted by serial dilution technique, which is illustrated in the following figure.



- The first test tube in the series contains 10 mL of undiluted sample. 1 mL of this sample is transferred into the second test tube, which already contains 9 mL of sterilized water. This procedure is followed to obtain larger and larger dilutions.

Multiple Tube Fermentation (MPN) Test:

Microorganisms belonging to the coliform group are considered to be "indicator" organisms for the presence of pathogenic bacteria in water. Coliform organism concentration in a water sample is determined by the MPN test. This test does not measure the actual number of coliform organisms in water. It actually assumes that the organisms in the water sample are randomly distributed and gives the number of organisms that is most likely to be present based on the probability of a particular combination of test results, using a statistical procedure. Two types of MPN tests are generally performed, the total coliform (TC) and the fecal coliform (FC) test. The FC test is generally more crucial, since the presence of fecal coliforms in water indicates mammalian fecal contamination of the water. This enhances the possibility of pathogens being present in the water sample.

Theory:

The statistical procedure used for MPN determination is described below. Let "n" be the number of organisms in a sample of volume "V" mL. Therefore the average number of organisms per mL of sample is n/V =λ. Note that this does not mean that every 1 mL aliquot from the original V mL sample must have λ organisms. In fact, any particular 1 mL aliquot may have 0, 1, 2 …n organisms. The probability that any 1 mL aliquot will have x organisms is given by poission distribution:

$$p(x) = \frac{\lambda^x . \exp(-\lambda)}{x!}$$

Thus the probability of any 1 mL sample having no organism and having one or more organism is given by: $p(x = 0) = \exp(-\lambda)$; and $p(x \neq 0) = 1 - \exp(-\lambda)$ respectively. The probability theory described above is used to calculate the value of most probable number (MPN) of organisms.

Total Coliform Test

The total coliform test is generally done in three phases, (a) presumptive, (b) confirmatory, and (c) completed. The sample is considered to be positive only if it shows positive results in all three phases. However, here we will only do the "presumptive" test.

Estimation of Most Probable Number (MPN) of Coliforms in Water

Procedure for Presumptive Test:

- Prepare lauryl-tryptose broth as per direction given on the label of the bottle containing the nutrient.
- Arrange test tubes to be used for the MPN test in a rack. Each group should have access to twelve test tubes.
- Using the water sample prepare three dilutions using the serial dilution procedure described earlier *(this will be done for you by the TAs and the will available in the lab. Please note the dilution values)*.
- To each of the 12 test tubes add approximately 10 mL of the broth.
- Put a durham tube in each of these tubes, ensuring that no air bubble is present in the durham tubes.
- Seal each tube with cotton wool. Then sterilize the tubes for 30 minutes in the sterilizer. *The sterilized test tubes containing the lauryl tryptose broth and durham tube will be available in the lab.*
- Add 1 mL portions of diluted water samples in nine tubes (each dilution in triplicate). Add 1 mL of a sterilized blank solution to the other three test tubes. To add the samples, take samples in sterilized 1 mL pipette, remove the cotton plug on the test tube and quickly add the sample before resealing with the same cotton plug. Use different pipets for different dilutions.
- Incubate at 35^{o}C for 24 hours. After this time visually examine for turbidity and gas production in the durham tube.
- Tubes exhibiting gas production are assumed to have given positive results indicating the presence of coliform organisms.

Procedure for Calculating MPN: An example problem

The presumptive MPN test for total coliforms (TC) was done with sample volumes of x, 0.1x and 0.01x mL. Some of the samples will be positive, and some negative. All tubes inoculated with dilution water must give negative results. Let the results for the three dilutions be as follows:

x mL	+	+	+
0.1x mL	+	-	-
0.01x mL	-	-	-

Calculate the MPN value.

Answer: The probability distribution for the above result is:

$$p(\lambda) = (1-\exp(-\lambda))^{3}.(\exp(-0.1\lambda))^{2}.(1-\exp(-0.1\lambda)).(\exp(-0.01\lambda))^{3}$$

The MPN value for the above sample is defined as that value of λ for which the value of $p(\lambda)$ is maximum. The solution can be obtained analytically, or by plotting the value of $p(\lambda)$ versus λ using a spreadsheet software and observing the value of λ corresponding to the maximum value of $p(\lambda)$.

Alternatively, the MPN value may be estimated using Thomas' formula:

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

Heterotrophic Plate Count Method

Theory:
The heterotrophic plate count (HPC) is a procedure for estimating the number of live heterotrophic bacteria in water. 1 mL of the sample (or diluted sample) is added to a petri dish. 10-12 mL of a nutrient broth (R2A agar in this case) is added to the petri dish and the contents mixed together. Once the plate hardens, the petri dish is incubated. It is assumed that each viable bacterium in the plate will multiply and form a colony. Thus a count of the number of colonies after suitable incubation time will result in the determination of the number of bacteria in the original water sample.

Procedure:

- Prepare R2A agar as per directions given on the label of the nutrient bottle *(This will be available to you in the laboratory).*
- Each group must possess twelve petri dishes.
- Prepare the twelve dishes (three sample dilutions and one blank, each in triplicate) as described below.
- One mL sample portion of contaminated water (or diluted water) is used for these tests. When discharging 1 mL sample portions hold the pipette at an angle of about 45^{o} with tip touching bottom of petri dish. Lift cover of petri-dish just high enough to insert the pipette.
- Maintain the previously sterilized and melted R2A agar in a water bath between 44 and 46^{o}C until used. Once the agar is taken out of the bath, the plating should be done in 20 minutes or less.
- Pour approximately 10-12 mL of the agar into each petri dish already containing the sample. Lift cover of the petri dish just high enough to pour the agar.
- As each plate is poured, mix melted medium thoroughly with the sample already in the petri dish.
- Let the plate solidify on a level surface. This will happen within 10 minutes.
- After the medium solidifies, invert plates and place in incubator. Incubate at 35^{o}C for 48 hours.

- Count colonies in selected plates promptly after incubation. Use an approved counting aid with magnification and illumination. Report results in terms of colony forming units CFUs/mL.
- Plates show colonies in the range 30 to 300 are the most reliable.

(A) Dry autoclaving of glassware for total coliform test

Sterilize glassware (pipettes) to be used in total coliform test by oven drying at 180^0C for 20 minutes.

(B) Preparation of lauryl-tryptose broth

Prepare lauryl-tryptose broth for presumptive test by suspending 1.78 g of lauryl-tryptose in 50 ml distilled water. To each of the 5 test tubes add approximately 10 mL of the broth. Put a durham tube in each of these tubes, ensuring that no air bubble is present in the durham tubes. Seal each tube with cotton wool.

(C) Wet autoclaving of test tubes for total coliform test

Sterilize the broth-filled test tubes for 20 minutes in the autoclave or sterilizer.

(D) Total Coliform Test

Perform total coliform test as per the procedure outlined earlier for presumptive test.

(E) Preparation of R2A agar broth for heterotrophic plate count test

Prepare R2A agar broth for heterotrophic plate count test by suspending 0.905 g of R2A agar in 50 ml distilled water. To dissolve completely heat it up to the boiling temperature.

(F) Heterotrophic plate count test

Perform heterotrophic plate count test as per the procedure outlined earlier for presumptive test.

Lab Exercise – 9

9A Understanding About Microbial Examination in Water

1. What are coliforms? Why do we test for coliforms in water? How is a positive coliform test interpreted? Are coliform bacteria harmful? Which coliforms are harmful? **(5 Marks)**
2. Explain what the most probable number (MPN) test is and how it is applied in water quality analysis. **(5 Marks)**
3. What does it mean if a water quality analysis returns a positive result for the presence of E. coli? What is the meaning of the term indicator organism? **(5 Marks)**
4. Early Monday morning you collect a water sample from a local beach to determine whether it is safe for swimming. You correctly set up an MPN test with the water sample. After the correct incubation time, you examine the tubes and discover a negative result in the 1 ml tube, but the 10 ml and 0.1 ml tubes are positive. **(10 Marks)**
    a. What should you tell your boss? Why?
    b. What could have caused the results you obtained? Be specific.
5. What is measured by Heterotrophic Plate Count Method? Differentiate between spread plate and pour plate technique. **(5 Marks)**

9B Estimation of Most Probable Number (MPN) of Coliforms in Water

1. The following MPN test results were obtained for three water samples. Determine the MPN/100 mL using (a) the Poisson distribution (b) the MPN tables, and (c) the Thomas equation. **(25 Marks)**

Sample Size mL	Number of Positive Tubes		
	Sample A	Sample B	Sample C
10	2	3	3
1	1	2	3
0.1	2	1	2

2. You are asked to perform a heterotrophic plate count (HPC) test on water from a dental chair. To do this, you collect water from the dental chair and inoculate a plate of R2A media with 100 µL. For identification, this plate is labeled "Plate A". You then make a dilution by taking 10 mL of the dental chair water and add it to 90 mL of sterile water. From this dilution, you inoculate another plate of R2A media with 100 µL of the dilution. For identification, the second plate is labeled "Plate B". After 24 h under incubation, you find Plate A has 120 colonies and Plate B has 2 colonies. What is the CFU/mL in the water obtained from the dental chair? **(20 Marks)**

3. Five ml of Bacterial Culture is added to 45 mL of sterile diluent. From this suspension, two serial, 1/100 dilutions are made, and 0.1 ml is plated onto Plate Count Agar from the last dilution. After incubation, 137 colonies are counted on the plate. Calculate CFU/mL of the original Sample. **(25 Marks)**