17MBU311 ENVIRONMENTAL MICROBIOLOGY – PRACTICAL (4H – 2C)

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 9 Hours

SCOPE

This paper provides a comprehensive overview of biogeochemical processes relevant to environmental scientists and engineers mediated by microorganisms.

OBJECTIVES

- To educate students about Environmental monitoring and environmental aspects of microbes.
- To impart a knowledge on Microbes and environment and ecological importance.

EXPERIMENTS

1. Analysis of soil - pH, moisture content, water holding capacity, percolation, capillary action.
2. Isolation of microbes (bacteria & fungi) from soil (28ºC & 45ºC).
3. Isolation of microbes (bacteria & fungi) from rhizosphere and rhizoplane.
4. Assessment of microbiological quality of water.
5. Determination of BOD of waste water sample.
6. Study the presence of microbial activity by detecting (qualitatively) enzymes (dehydrogenase, amylase and urease) in soil.
7. Isolation of *Rhizobium* from root nodules.

EXPERIMENT-1

Analysis of soil - pH, moisture content, water holding capacity, and percolation

Aim

To study moisture content, pH, water holding capacity and water percolation rate of different soil samples.

pH

The soil pH is the negative logarithm of the active hydrogen ion (H+) concentration in the soil solution. It is the measure of soil acidity or neutrality. It is a simple but very important estimation for soils, since soil pH influences to a great extent the availability of nutrients to crops. It also affects microbial population in soils.

Material Required

Soil samples, watch glass, test tubes, funnel, beakers, filter paper, weighing balance, dropper, pH paper booklet, tile.

Procedure:

Preparation of soil solution and pH measurement

Weigh10.0g of soil samples (roadside, garden soil, humus rich soil and riverside soil) in separate watch glass and dissolve it separate beakers containing 50 ml water to make soil solution.

Take a funnel, place a filter paper in it and keep it on test tube.

Take roadside soil solution and filter the solution through the filter paper and collect the filtrates in a test tube.

Repeat the same procedure for other samples with fresh filter papers.

Take a pH paper booklet.

Tear pH paper strips from the booklet and place 4 strips on the tile.

Using a dropper, take few drops of roadside soil solution from the test tube.

Put 1 to 2 drops of solution on the first pH strip on the tile.

Using fresh droppers, do the same procedure for garden soil, humus rich and riverside soil.

Wait for few minutes and note the colour and compare with the colour chart given on the broad range indicator paper and get the estimate of pH of the soil solutions.

Observation

Colour change was noted in the pH strip after adding soil solution, and compared with the colour chat given on the pH indicator strip.

Result

The roadside soil has pH 7, garden soil and humus rich soil have ph 6 and riverside soil has pH 8.

Moisture Content of Soil

Gravimetric method of moisture estimation is most widely used where the soil sample is placed in an oven at 105°C and dried to a constant weight. The difference in weight is considered to be water present in the soil sample.

Materials Required

Soil samples, weighing balance, aluminium moisture box, oven and tong

Procedure to determine moisture content in soil by oven drying method

Clean the aluminium moisture box, dry it and weigh it with the lid (Weight „W_1").

Take the required quantity of the wet garden soil in the container and weigh it with the lid (Weight „W_2").

Place the container, with its lid removed, in the oven till its weight becomes constant (normally for 24 hrs.).

When the soil has dried, remove the container from the oven, using tongs.

Find the weight „W_3"of the container with the lid and the dry soil sample.

An average of three determinations should be taken.

Repeat the same procedure for roadside soil.

Record the initial and final weights of each sample and the difference between initial and final weights in the form of a table.

Formula to calculate the moisture content of the soil

Water content W= $(W_2-W_3) / (W_3-W_1)$ x100

S.No.	Description- Garden soil	Determination No.		
		I	II	III
1.	Weight of Empty Container (W_1) in g	20.12	20.08	20.00
2.	Weight of Container +Wet Soil (W_2) in g	44.12	44.11	46.10
3.	Weight of Empty Container + Dry Soil (W_3) in g	41.18	41.16	43.01
Calculation				
1.	Weight of water = W_2-W_3	2.94	2.95	3.09
2.	Weight of Solid = W_3-W_1	21.06	21.08	23.01
3.	Water content W= $(W_2-W_3) / (W_3-W_1)$ x100	13.96	13.99	13.43
Average Value		**13.79%**		

S.No.	Description- Roadside soil	Determination No.		
		I	II	III
1.	Weight of Empty Container (W_1) in g	20.12	20.08	20.00
2.	Weight of Container +Wet Soil (W_2) in g	43.10	43.09	43.96
3.	Weight of Empty Container + Dry Soil (W_3) in g	42.50	42.39	43.00
Calculation				
1.	Weight of water = W_2-W_3	0.6	0.7	0.96
2.	Weight of Solid = W_3-W_1	22.38	22.31	23.00
3.	Water content W= (W_2-W_3) / (W_3-W_1) x100	2.6	3.13	4.17
Average Value		**3.33%**		

Observation

Garden soil shows higher difference between initial and final weight indicating higher moisture content in the garden soil than the roadside soil.

Result

The moisture content of the garden soil is 13.79% and the moisture content of the road side soil is 3.33%.

Water Holding Capacity of Soil

Veihmeyer and Hendrickson (1931) defined the field capacity or the water holding capacity as the amount of water held in the soil after the excess gravitational water has drained away and after the rate of downward movement of water has materially ceased. One of the main functions of soil is to retain water and make it available for the plant to access. All of the water in the soil is not available to plants.

Materials Required

Soil samples, round tin box vessel with perforated bottom, mortar and pestle, filter paper, weighing balance, small glass rods, Petri dish.

Procedure

Take watch glass containing garden soil and put it into a mortar and grind it using a pestle into fine powder.
Take a filter paper and place it in the bottom of the perforated tin box.
Weigh the tin along with the filter paper and note its weight.

Transfer the fine powdered soil sample into the tin box and tap the box gently several times, so that soil is compactly formed a layer at the top.

Weigh the tin box along with soil sample and note its weight.

Take a Petri dish filled with water and two small glass rods and place them parallel to and at a small distance from each other.
Place the soil filled tin on the two glass rods in such a manner that its bottom is in contact with water.

Leave the set up undisturbed till water appears on the upper surface of the soil. Wait till entire soil surface is wet.
Remove the tin and allow all the gravitational water to flow out from the bottom.
When no more water percolates, wipe the bottom to dry it with a filter paper.
Weigh them again and note its weight.

Record all the values into the table and calculate the percentage of water holding capacity of the soil.

Calculation

Soil Sample	Weight of tin + Filter Paper (A in grams)	Weight of tin + Filter Paper +	Weight of tin + Filter Paper + Wet	Weight of Dry Soil B-A= C grams	Weight of Wet Soil D-A= E grams	Mass of water absorbed by soil E-C = N grams	% of Water Holding Capacity

		Soil Sample (B in grams)	soil (D in grams)				
Garden Soil	65.37	95.86	115.19	30.49	49.82	18.92	18.92%
Roadside soil	65.37	95.86	104.89	30.49	39.52	9.03	9.03%

Observation

Garden soil shows higher difference between initial and final weight indicating higher water holding capacity in the garden soil than the roadside soil.

Result

The water holding capacity of the garden soil is 18.92% and the water holding capacity of the road side soil is 9.03%.

Water percolation capacity of soil

When water is added to soil, the water we added has to pass through in between gaps or channels of soil particles. The speed at which the water flows through the column of soil is known as percolation rate or percolation of water depends on the particle size of the given soil sample

Materials Required:

Soil samples, funnels, cotton, conical flask, beakers and coloured water.

Procedure

Take a funnel and plug its nozzle with of cotton, place it on conical flask and add 20 gram of samples, each in a separate setup (sand and clay).

Now pour 50 ml of coloured water over each type of soil and observe the amount of water percolated through the funnel.

Measure the volume of water which sieves through the funnel in unit time.

Percolation rate of water can be calculated using the formula

$$Percolation\ rate\ (mL/min) = \frac{Amount\ of\ water\ in\ mL}{Time\ taken\ to\ percolate\ in\ minute}$$

Soil Sample	Amount of water in mL	Time taken to percolate in Minute	Percolation rate (mL/Min)
Sand	35 mL	10 Min	(3.5 mL/Min)
Clay	-	-	-

Observation

The percolation rate of water in sandy soil is fastest and in clayey soil is slowest.

Result

Sand has the highest percolation rate of (3.5 mL/Min), while clay did not percolate any water so clay has the highest retention capacity.

EXPERIMENT-2

Isolation of microbes (bacteria & fungi) from soil

Aim

To isolate bacteria and fungi present in soil sample.

Materials Required

Petri dishes, 1-ml pipettes 10-ml pipette, test tubes, conical flasks, 500 ml beaker, glass spreader, stainless steel pipette case, ethyl alcohol, 0.1N hydrochloric acid (HCI), 0.1N sodium hydroxide (NaOH), distilled water, nutrient agar, soil sample, pH meter, pestle and mortar, bunsen burner, autoclave, incubator, laminar flow chamber, turntable and Quebec colony counter.

Background

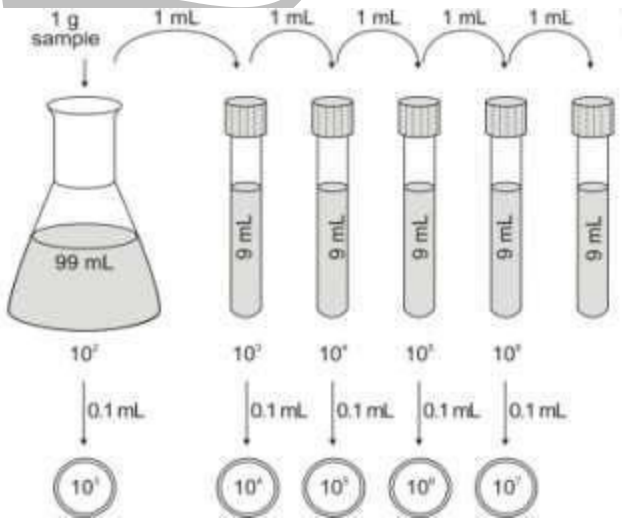
Soil contains myriads of microorganisms, including bacteria, fungi, protozoa, algae, and viruses. The most prevalent are bacteria, including the mold like actinomycetes, and fungi: It is essential to bear in mind that the soil environment differs from one location to another and from one period of time to another. Therefore, factors such as moisture, pH, temperature, gaseous oxygen content, and organic and inorganic composition of soil are crucial in determining the specific microbial flora of a particular sample. Just as the soil differs, microbiological methods used to analyze soil also vary. A single technique cannot be used to count all the different types of microorganisms present in a given soil sample because no one laboratory cultivation procedure can provide all the physical and nutritional requirements necessary for the growth of a greatly diverse microbial population. In this experiment, only the relative numbers of bacteria and fungi are determined. The method used is the serial dilution–agar plate procedure. Different media are employed to support the growth of these microorganisms potato dextrose agar for the isolation of fungi, and nutrient agar for the isolation of bacteria. The potato dextrose agar was supplemented with 10 3g of chlortetracycline per millilitre of medium to inhibit the growth of bacteria.

Procedure

1. Add 99 mL of deionised water in a conical flask and 9 ml of deionised water was pipetted into each of the test tubes, their mouths are cotton-plugged, covered with paper and tied with thread or rubber band. These are used as diluents for serial dilution.
2. The number of Petri dishes and accordingly the amount of medium to be used is calculated depending on the number of replications and dilutions required. The ready-made powder for nutrient agar medium

and potato dextrose is weighed and dissolved in of distilled water in conical flask by shaking and swirling.

3. The pH is determined using a pH paper or pH meter and adjusted to 7.0/5.5 using 0.1N HCl if it is more or using 0.1N NaOH if it is less. Then, it is cotton-plugged, covered with paper and tied with thread or rubber band.
4. The conical flask containing 99 ml of deionised water , the test tubes containing 9 ml of deionised water each and the conical flask containing of nutrient agar medium are sterilized at 121°C (15 psi pressure) for 15 minutes in an autoclave.
5. After sterilisation, the sterilised materials are removed from the autoclave and allowed to cool for some time, without allowing the medium to solidify.
6. To prepare agar plates, before the sterilised nutrient agar/potato dextrose agar medium cools and solidifies, in warm molten condition, it is poured aseptically into the sterilised petri dishes (approximately 20 ml each), so that the molten medium covers the bottom of the Petri dishes completely. Then, the plates are covered with their lids and allowed to cool, so as to solidify the medium in them.
7. To begin the procedure, weigh out 1 g of soil sample and add to 99 mL of deionised water. Shake the suspension well, and label as 10^{-2}.
8. Before the soil settles, remove 1 mL of the suspension with a sterile pipette and transfer it to 9 mL deionised water blank. Vortex thoroughly, and label as 10^{-3}.
9. Repeat this dilution step, each time with 1 mL of the previous suspension and 9-mL deionised water blank. Label these sequentially, this results in serial dilutions of 10^{-2} through 10^{-7} the final dilution



10. To grow bacterial colonies, take pre-prepared nutrient agar plates and label them, using sterile 1 ml pipettes, add 1 ml of each dilution into each Petri dish as indicated 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions to be used for enumeration of bacteria. With an alcohol-dipped and flamed L- rod, spread the culture over the entire agar surface. Re-flame the spreader and repeat the process for each dilution into each Petri dish.

11. To grow fungi, take pre-prepared potato dextrose agar plates and label them, using sterile 1 ml pipettes, add 1 ml of each dilution into each Petri dish as indicated 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions to be used for enumeration of fungi.
12. Incubate the plates in an inverted position at 28°C. Perform colony counts on nutrient agar plate cultures in 2 to 3 days and on the remaining agar plate cultures in 4 to 7 days. An un-inoculated agar plate is incubated as control to ensure proper sterilisation to show no growth on it.
13. Using an electronic colony counter or a Quebec colony counter and a mechanical hand counter, observe all the colonies on each nutrient agar plate 2 to 3 days after incubation begins and remaining PDA agar plate cultures in 4 to 7 days. Plates with more than 300 colonies cannot be counted and should be designated as too numerous to count (TNTC); plates with fewer than 30 colonies should be designated as too few to count (TFTC). Count only plates with between 30 and 300 colonies.

Observation

After 2-3 days of incubation, the nutrient agar plates showed different colonies with white, cream, or yellow in colour, and fairly circular in shape. Where as in PDA plates growth was observed at 4 to 7 days whitish grey, with fuzzy edge colonies was observed.

Calculation

The number of colonies of bacteria/fungi on the plates is counted directly or with the help of a Quebec colony counter. From this, the numbers of bacteria/fungi present per gram or ml of the original sample is calculated. The number of organisms per ml of original culture is calculated by multiplying the number of colonies counted by the dilution factor:

Number of cells per ml = Number of colonies x Dilution factor

Organism	Dilution	Number of Colonies	Organisms per Gram of Soil
Bacteria	10^{-4}	> 300	too many to count
	10^{-5}	216	216×10^5
	10^{-6}	< 30	too less to count

Fungi	10^{-2}	> 300	too many to count
	10^{-3}	98	98×10^3
	10^{-4}	< 30	too less to count

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Calculation bacterial colonies

Colonies per plate = 216

Dilution factor = 10^5 (1, 00,000)

Volume of dilution added to plate = 0.1 ml

216 x1, 00,000 = 21,600,000 or (216×10^5) cells/0.1 ml

To calculate for 1 mL

21,600,000 x 10 = 21, 60, 00,000

(216×10^6) CFUs/g

Calculation fungal colonies

Result

Colonies per plate = 98 Dilution factor = 10^3 (1,000)

Volume of dilution added to plate = 0.1 ml

98 x1, 000 = 98,000 or (98 x 10^3) cells/0.1 ml

To calculate for 1 mL

98,000 x 10 = 9, 80,000

(98 x 10^4) CFUs/g

A gram of soil contains viable culturable bacteria of 216 x 10^6 CFU/g and fungi of 98 x 10^4 CFU/g by serial dilution and plating technique.

EXPERIMENT-3

Isolation of microbes (bacteria & fungi) from rhizosphere and rhizoplane

Aim

To isolate bacteria and fungi from rhizosphere and rhizoplane soil

Materials Required

Petri dishes, 1-ml pipettes 10-ml pipette, test tubes, conical flasks, 500 ml beaker, glass spreader, stainless steel pipette case, ethyl alcohol, 0.1N hydrochloric acid (HCI), 0.1N sodium hydroxide (NaOH), distilled water, soil extract agar, rhizosphere soil, rhizoplane soil, pH meter, bunsen burner, autoclave, incubator, laminar flow chamber, turntable.

Background

The rhizosphere, the narrow zone of soil that surrounds and is influenced by plant roots, is home to an overwhelming number of microorganisms and is considered to be one of the most dynamic interfaces on Earth. Organisms that are present in the rhizosphere microbiota can have profound effects on the growth, nutrition and health of plants in agro-ecosystems. Rhizosphere microbiotas can also directly and/or indirectly affect the composition and biomass of plant communities in natural ecosystems. Numerous organisms contribute to these processes, leading to countless interactions between plants, antagonists and mutualistic symbionts, both below ground and above ground. Bacteria, fungi (including arbuscular mycorrhizal fungi (AMF)), oomycetes, viruses and archaea that live in the rhizosphere are attracted by and feed on rhizodeposits - that is, nutrients, exudates, border cells and mucilage released by the plant root. Roots release low-molecular-mass compounds (that is, sugars, amino acids and organic acids), polymerized sugar (that is, mucilage), root border cells and dead root cap cells. These rhizodeposits are used as carbon sources by soil microorganisms. Rhizodeposits also contain secondary metabolites, such as antimicrobial compounds, nematicides and flavonoids, which are involved in establishing symbiosis or in warding off pathogens and pests.

Procedure

1. A block of soil encompassing the complete root system of a seeding is cut out and placed in a clean container.
2. Gently crush the block of soil to recover the root system in such a way that some soil still adheres to the roots even after gentle shaking.
3. Soil adjacent to the root segments, at 1-5 mm from the root surface, was shaken off and defined as rhizosphere soil. Soil farther than 1 cm from the roots was collected and considered as rhizoplane soil.

Collected soils were sieved in a 1mm mesh removing root hair as much as possible.

4. Ten fold serial dilution of rhizosphere and rhizoplane soil was prepared separately by dissolving 1g of soil in 9 ml of sterile distilled water to get 10^{-1} dilution. Repeat this dilution step, each time with 1 mL of the previous suspension and 9-mL deionised water blank. Label these sequentially, this results in serial dilutions of 10^{-2} through 10^{-4} the final dilution.
5. The ingredients of soil extract agar medium is weighed and dissolved in of distilled water in conical flask by shaking and swirling and sterilized by autoclaving.

Composition Ingredients	**Gms/Litre**		
Glucose	1.000	Dipotassium phosphate	0.500
Soil extract	17.750		
Agar	15.000		
Final pH (at 25°C)	6.8±0.2		

6. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.
7. 0.1 ml of aliquot was plated into soil extract agar plates for bacteria at10^{-4} dilution and fungi at10^{-2} dilution (with kanamycin antibiotic at 50 μg^{ml}). The plates were incubated at room temperature for 72 hours and the number of bacterial and fungal colonies was counted.

Observation

After 2-3 days of incubation, bacterial plates showed different colony morphology with pigmented colonies white, cream, or yellow in colour, and fairly circular in shape. Whereas in fungal plates growth was observed at 4 to 7 days whitish grey, with fuzzy edge colonies.

Result

The bacterial and fungal population in the rhizosphere and rhizoplane soil was 5 x 10^2cfu/g and 3 x 10^3 cfu/g and 2.9 x 10^2cfu/g and 1.5 x 10^3 cfu/g at 3 to 6 days of incubation respectively.

EXPERIMENT-4

Assessment of microbiological quality of water

The importance of potable (drinking) water supplies cannot be overemphasized. With increasing industrialization, water sources available for consumption and recreation have been adulterated with industrial as well as animal and human wastes. As a result, water has become a formidable factor in disease transmission. Polluted waters contain vast amounts of organic matter that serve as excellent nutritional sources for the growth and multiplication of microorganisms. The presence of nonpathogenic organisms is not of major concern, but intestinal contaminants of fecal origin are important. These pathogens are responsible for intestinal infections such as bacillary dysentery, typhoid fever, cholera, and paratyphoid fever.

The World Health Organization (WHO) estimates that 1.7 million deaths per year result from unsafe water supplies. Most of these are from diarrheal diseases, and 90% of these deaths occur in children living in developing countries where sanitary facilities and potable water are at a minimum. The WHO indicates that about million deaths annually are caused by danger- ous waterborne enteric bacterial pathogens such as *Shigella dysenteriae, Campylobacter jejuni, Salmonella typhi,* and *Vibrio cholerae.*

In addition to bacterial infections, unsafe water supplies are responsible for numerous parasitologi- cal infections, including helminth diseases such as schistosomiasis and especially guinea worm *(Dracunculus medinensis),* which infects about 200 million people worldwide each year. Intestinal, hepatic, and pulmonary flukes such as *Fasciolopsis buski, Clonorchis sinensis,* and *Paragonimus westermani* are responsible for human infection and are all associated with unsafe water and sanita- tion. The parasitic protozoa *Entamoeba histolytica, Giardia intestinalis* (formerly called *G. lamblia*), and *Balantidium coli* are just a few of the protozoa responsible for major diarrheal disease in humans.

Although waterborne infections occur in the United States, their incidence in comparison to the rest of the world is much lower, and they occur sporadically. This can be attributed to the diligent attention given to our water supplies and sewage disposal systems.

Analysis of water samples on a routine basis would not be possible if each pathogen required detection. Therefore, water is examined to detect *Escherichia coli,* the bacterium that indicates fecal pollution. Since *E. coli* is always present in the human intestine, its presence in water alerts public health officials to the possible presence of other human or animal intestinal pathogens. However, in the tropics and subtropics it is not considered a reliable indicator of fecal pollution because the soil in these regions naturally contains high levels of *E. coli.* Therefore, *E. coli* is present in the water anytime there is surface runoff. Both qualitative and

quantitative methods are used to determine the sanitary condition of water.

Principle

The three basic tests to detect coliform bacteria in water are presumptive, confirmed, and completed.The tests are performed sequentially on each sample under analysis. They detect the presence of coliform bacteria (indicators of fecal contamination), the gram-negative, non–spore-forming bacilli that ferment lactose with the production of acid and gas that is detectable following a 24-hour incubation period at 37°C.

The PresumptiveTest

The presumptive test is specific for detection of coliform bacteria. Measured aliquots of the water to be tested are added to a lactose fermentation broth containing an inverted gas vial. Because these bacteria are capable of using lactose as a carbon source (the other enteric organisms are not), their detection is facilitated by the use of this medium. In this experiment, you will use lactose fermentation broth containing an inverted Durham tube for gas collection.

Tubes of this lactose medium are inoculated with 10-ml, 1-ml, and 0.1-ml aliquots of the water sample. The series consists of at least three groups, each composed of five tubes of the speci- fied medium. The tubes in each group are then inoculated with the designated volume of the water sample, as described under "Procedure: Lab One." The greater the number of tubes per group, the greater the sensitivity of the test. Development of gas in any of the tubes is *presumptive* evidence of the presence of coliform bacteria in the sample.

The presumptive test also enables the microbiologist to obtain some idea of the number of coliform organisms present by means of the **most probable number (MPN) test.** The MPN is esti- mated by determining the number of tubes in each group that show gas following the incubation period.

The ConfirmedTest

The presence of a positive or doubtful presumptive test immediately suggests that the water sample is nonpotable. Confirmation of these results is necessary because positive presumptive tests may be the result of organisms of noncoliform origin that are not recognized as indicators of fecal pollution.

The **confirmed test** requires that selective and differential media such as eosin–methylene blue (EMB) or Endo agar be streaked from a positive lactose broth tube obtained from the presumptive test. The nature of the differential and selective media was discussed in Experi- ment 15 but is reviewed briefly here. Eosin–methylene blue contains the dye methylene blue, which inhibits the growth of gram-positive organ- isms. In the

presence of an acid environment, EMB forms a complex that precipitates out onto the coliform colonies, producing dark centers and a green metallic sheen. The reaction is character- istic for *Escherichia coli*, the major indicator of fecal pollution. Endo agar is a nutrient medium containing the dye fuchsin, which is present in the decolorized state. In the presence of acid pro- duced by the coliform bacteria, fuchsin forms a dark pink complex that turns the *E. coli* colonies and the surrounding medium pink.

The CompletedTest

The **completed test** is the final analysis of the water sample. It is used to examine the coliform colonies that appeared on the EMB or Endo agar plates used in the confirmed test. An isolated colony is picked up from the confirmatory test plate and inoculated into a tube of lactose broth and streaked on a nutrient agar slant to perform a Gram stain. Following inoculation and incubation, tubes showing acid and gas in the lactose broth and presence of gram-negative bacilli on micro- scopic examination are further confirmation of the presence of *E. coli*, and they are indicative of a positive completed test.

Materials Required

Cultures

Water samples from sewage plant, pond, and tap. Lab Two: One 24-hour-old positive lactose broth culture from each of the three series from the presumptive test. Lab Three: One 24-hour coliform-positive EMB or Endo agar culture from each of the three series of the confirmed test.

Media

Lab One (per designated student group): 15 double- strength lactose fermentation broths (LB2X) and 30 single-strength lactose fermentation broths (LB1X). Lab Two (three each per designated student group): eosin–methylene blue agar plates or Endo agar plates. Lab Three (three each per designated student group): nutrient agar slants and lactose fermentation broths.

Reagents

Lab Three: Crystal violet, Gram‟s iodine, 95% ethyl alcohol, and safranin.

Equipment

Lab One: Bunsen burner, 45 test tubes, test tube rack, sterile 10-ml pipettes, sterile 1-ml pipettes, sterile 0.1-ml pipettes, mechanical pipetting device, and glassware marking pencil. Lab Two: Bunsen burner, glassware marking pencil, and in- oculating loop. Lab Three: Bunsen burner, staining tray, inoculating loop, lens paper, bibulous paper, microscope, and glassware markingpencil.

Presumptive Test

Set up three separate series consisting of three groups, a total of 15 tubes per series, in a test tube rack; for each tube, label the water source and volume of sample inoculated as illustrated.

Mix sewage plant water sample by shaking thoroughly.

Flame bottle and then, using a 10-ml pipette, transfer 10-ml aliquots of water sample to the five tubes labeled LB2X-10 ml.

Flame bottle and then, using a 1-ml pipette, transfer 1-ml aliquots of water sample to the five tubes labeled LB1X-1 ml.

Flame bottle and then, using a 0.1-ml pipette, transfer 0.1-ml aliquots of water sample to the five tubes labeled LB1X-0.1 ml.

Repeat Steps 2 through 5 for the tap and pond water samples.
Incubate all tubes for 48 hours at 37°C.

Presumptive Test

Examine the tubes from your presumptive test after 24 and 48 hours of incubation. Your results are positive if the Durham tube fills 10% or more with gas in 24 hours, doubtful if gas develops in the tube after 48 hours, and nega- tive if there is no gas in the tube after 48 hours. Record your results in the Lab Report.

Determine the MPN using Table, and record your results in the Lab Report.

Confirmed Test

Label the covers of the three EMB plates or the three Endo agar plates with the source of the water sample (sewage, pond, and tap).

Using a positive 24-hour lactose broth culture from the sewage water series from the presumptive test, streak the surface of one EMB or one Endo agar plate, as described in Experiment 3, to obtain discrete colonies.

Repeat Step 2 using the positive lactose broth cultures from the pond and tap water series from the presumptive test to inoculate the remaining plates.

Incubate all plate cultures in an inverted position for 24 hours at 37°C.

Confirmed Test

Examine all the plates from your confirmed test for the presence or absence of E. coli colonies (refer to the description of the con- firmed test in the experiment introduction, and see Figure 15.2 for an illustration of E. coli growth on EMB agar). Record your results in the Lab Report.

Based on your results, determine whether each of the samples is potable or nonpotable. The presence of E. coli is a positive confirmed test, indicating that the water is nonpotable. The absence of E. coli is a negative test, indicating that the water is not contaminated with fecal wastes and is therefore potable. Record your results in the Lab Report.

Completed Test

Label each tube of nutrient agar slants and lactose fermentation broths with the source of its water sample.

Inoculate one lactose broth and one nutrient agar slant with a positive isolated E. coli colony obtained from each of the experimental water samples during the confirmed test.

Incubate all tubes for 24 hours at 37°C.

Completed Test

Examine all lactose fermentation broth cultures for the presence or absence of acid and gas. Record your results in the Lab Report.

Prepare a Gram stain, using the nutrient agar slant cultures of the organisms that showed a positive result in the lactose fermentation broth

Examine the slides microscopically for the presence of gram-negative short bacilli, which are indicative of E. coli and thus nonpotable water. In the Lab Report, record your results for Gram stain reaction and morphology of the cells.

Observations and Results

PresumptiveTest

	GAS																	
	LB2X-10					LB1X-1					LB1X-0.1					95% Probability		
	Tube					Tube					Tube					Reading	MPN	Range
Water Sample	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**			
Sewage																		
Pond																		
Tap																		

ConfirmedTest

Water Sample	COLIFORMS		**Potable**	**Nonpotable**
	EMB Plate	**Endo Agar Plate**		
Sewage				
Pond				
Tap				

CompletedTest

Water Source	Lactose Broth A/G (+) or (−)	GRAM STAIN	POTABILITY	
		Reaction/Morphology	Potable	Nonpotable
Sewage				
Pond				
Tap				

TABLE TheMPN Indexper100mlforCombinations of Positive and Negative Presumptive Test Results When Five 10-ml, Five 1-ml,andFive0.1-mlPortionsofSampleAreUsed

NUMBER OF TUBES WITH POSITIVE RESULTS				95 % CONFIDENCE LIMITS		NUMBER OF TUBES WITH POSITIVE RESULTS				95 % CONFIDENCE LIMITS	
FIVE OF 10 ML EACH	FIVE OF 1 ML EACH	FIVE OF 0.1 ML EACH	MPN INDEX PER 100 ML	LOWER	UPPER	FIVE OF 10 ML EACH	FIVE OF 1 ML EACH	FIVE OF 0.1 ML EACH	MPN INDEX PER 100 ML	LOWER	UPPER
0	0	0	<2	0	6	4	2	1	26	7	67
0	0	1	2	<0.5	7	4	3	0	27	9	78
0	1	0	2	<0.5	7	4	3	1	33	9	78
0	2	0	4	<0.5	11	4	4	0	34	11	93
1	0	0	2	0.1	10	5	0	0	23	7	70
1	0	1	4	0.7	10	5	0	1	31	11	89
1	1	0	4	0.7	12	5	0	2	43	14	100
1	1	1	6	1.8	15	5	1	0	33	10	100
1	2	0	6	1.8	15	5	1	1	46	14	120

2	0	0	5	<0.5	13	5	1	2	63	22	150
2	0	1	7	1	17	5	2	0	49	15	150
2	1	0	7	1	17	5	2	1	70	22	170
2	1	1	9	2	21	5	2	2	94	34	230
2	2	0	9	2	21	5	3	0	79	22	220
2	3	0	12	3	28	5	3	1	110	34	250
3	0	0	8	2	22	5	3	2	140	52	400
3	0	1	11	4	23	5	3	3	180	70	400
3	1	0	11	5	35	5	4	0	130	36	400
3	1	1	14	6	36	5	4	1	170	58	400
3	2	0	14	6	36	5	4	2	220	70	440
3	2	1	17	7	40	5	4	3	280	100	710
3	3	0	17	7	40	5	4	4	350	100	710
4	0	0	13	4	35	5	5	0	240	70	710
4	0	1	17	6	36	5	5	1	350	100	1100
4	1	0	17	6	40	5	5	2	540	150	1700
4	1	1	21	7	42	5	5	3	920	220	2600
4	1	2	26	10	70	5	5	4	1600	400	4600
4	2	0	22	7	50	5	5	5	≥2400	700	– ––

EXPERIMENT-5

Determination of BOD of waste water sample

Biological Oxygen Demand (BOD_5)

BOD_5 is an experimental test in which standardized laboratory procedures are used to determine the relative oxygen requirement of waste water (APHA, 1989).

Principle

The method consists of filling the sample, to overflowing, in an airtight bottle of the specified size and incubating it at the specified temperature for 5 days. Dissolved oxygen (DO) is measured initially and after incubation, and the BOD is computed from the difference between initial and final DO. Because the initial DO is determined immediately after the dilution is made, all oxygen uptake, including that occurring during the first 15 minutes, is included in the BOD measurement.

Sampling and Storage

Samples for BOD analysis may degrade significantly during the storage between collection and analysis resulting in low BOD values. To minimize reduction of BOD, sample is analysed promptly or by cooling it to near-freezing temperature during storage. However, even at low temperature, the holding time is kept to a minimum. (Chilled samples are warmed to 20°C before analysis).

Apparatus

Incubation bottles

300ml capacity clean bottles were used for sampling, water seal was used as a precaution against drawing air into the dilution bottle during incubation. Care was taken to obtain satisfactory water seals by adding water to the flared mouth of special BOD bottles.

BOD incubator

Thermostatically controlled BOD incubator was used at 20°C. Care taken to exclude all the light to prevent the possibility of photosynthetic production of DO, by algae in the sample.

Reagents

1. **Phosphate – buffer**

   Dihydrogen Orthophosphate (KH_2PO_4), 8.5 gm, 21.75 gm of potassium monohydrogen orthophosphate (K_2HPO_4), 33.4 gm of disodium hydrogen phosphate ($Na_2HPO_4.7H_2O$) and 1.7 gm ammonium chloride (NH_4C1) was dissolved in 500 ml of distilled water and it was made

upto 1000 ml. The pH was adjusted to 7.2.

2. **Magnesium Sulphate**

   Magnesium sulphate ($MgSO_4.7H_2O$) 22.5 gm was dissolved in distilled water and made upto 1000 ml.

3. **Calcium Chloride**

   Anhydrous calcium chloride ($CaCl_2$) 27.5 gm was dissolved in distilled water and made upto 1000 ml with distilled water.

4. **Ferric Chloride Solution**

   Ferric Chloride ($Fe0l_3.6H_2O$) 0.25 gm was dissolved in distilled water and made upto 1000 ml.

5. **Manganous Sulphate**

   Manganous sulphate ($MnSO_4.4H_2O$) 480 gm was dissolved in 1000 ml of distilled water.

6. **Alkaline Iodide**

   Potassium hydroxide (KOH) 700 gm and 150 gm of Potassium iodide were dissolved in little water separately and both were mixed and made upto 1000 ml distilled water.

7. **Concentrated sulphuric acid (H_2SO_4)**

8. **Starch Indicator (1%)**

   One gm of starch was dissolved in 100 ml of boiling distilled water. Sodium thiosulfate ($Na_2S_2O_3.5H_2O$) (0.025N) $Na_2S_2O_3.5H_20$ 6.205 was dissolved in previously boiled distilled water and was made upto 1000 ml. This solution was stored in a brown bottle with the addition of 5 ml chloroform to serve as preservative.

Procedure

1. **Preparation of dilution water**

   Ten litre of distilled water was taken in a suitable container. To this, 1% of phosphate buffer, magnesium sulphate, calcium chloride and ferric chloride were added into dilution water. Before use, the dilution water temperature was brought to 20°C. This water was aerated using air pump.

2. **Seeding**

   It is necessary to have a population of microorganisms capable of oxidizing the bio-degradable organic matter in the sample. Domestic waste water, un-chlorinated or otherwise un-disinfected effluent from biological waste treatment plants, serve as the seed source.

3. Dilution

The dilutions were prepared in graduated cylinders and then transferred to BOD bottles.

4. Determination of initial DO

If the sample contains materials that react rapidly with DO, initial DO was determined after filling BOD bottles with diluted sample.

5. Dilution water blank

A dilution blank was used for a rough check on the quality of unseeded dilution water and cleanliness of incubation bottles.

6. Incubation

BOD bottles containing desired dilutions, seed control and dilution water blank were incubated at 20°C ± 1°C.

7. Determination of final DO

After 5 days of incubation, DO of the samples and dilution blank were determined.

Calculation

$$BOD_5 \text{ mg/L} = \frac{(D_1-D_2) - (B_1-B_2)\, f}{P}$$

Where

D_1 = DO of diluted sample immediately after preparations, (mg/L).

D_2 = DO of diluted sample after 5 days incubation at 20°C, (mg/L).

B_1 = DO of seed control before incubation, (mg/L).

B_2 = DO of seed control after incubation, (mg/L).

f = Ratio of seed in diluted sample to seed in seed control

$$= \frac{\% \text{ seed in } D_1}{\% \text{ seed in } B_1}$$

Seed correction = $(B_1 - B_2)\, f$

P = decimal volumetric fraction of sample.

EXPERIMENT-6

Study the presence of microbial activity by detecting (qualitatively) dehydrogenase enzyme in soil.

Aim

To demonstrate the dehydrogenase activity associated with soil microorganisms.

Materials Required

Thermostatic incubator (30°C)

Microplate reader / Spectrophotometer (wavelength of 3=485 nm)

Methanol

Screw cap tubes with tightly fitting lids

Weighing balance

Deionized water

1ml and 10 ml pipettes

Soil Sample

Three percent solution of 2, 3, 5-triphenyltetrazolium chloride (TTC): Dissolve 3 g of TTC in about 80 mL of water and finally make up the volume to 100 mL with water.

Triphenylformazan (TPF) standard solution: Dissolve 100 mg of TPF in about 80 mL of methanol and adjust the volume to 100 mL with methanol, mix thoroughly.

Principle

Dehydrogenase activity was determined according to the Thalmann (1968) method modified by Alef and Nannipieri (1995). The method allows to determine the effect of exogenous organic matter on activity of soil dehydrogenases. The method consists in incubation of soil with artificial electron acceptor, the colourless and water-soluble 2, 3, 5-triphenyltetrazolium chloride (TTC), which is reduced due to the action of dehydrogenases to the red-colored triphenyl formazan (TPF), insoluble in water. Replacing oxygen and other natural acceptors, TTC intercepts electrons and protons which were detached by dehydrogenases from oxidized

organic compounds. After the incubation, formazan is extracted from the soil with alcohol, and determined colorimetrically.

Soil dehydrogenase activity (DHA) is the result of the activity of different dehydrogenase enzymes involved in the oxidative metabolism of viable microbial cells. Under aerobic soil conditions, oxygen (O_2) is a final acceptor of electrons and protons detached by dehydrogenases, while under anaerobic conditions the function of the terminal acceptor is taken over by oxidized inorganic forms, such as nitrate, Mn (IV), Fe (III), sulfate, or by some organic compounds. Irrespective of the soil aeration, dehydrogenases are an element of the microbial respiratory metabolism related to the ATP synthesis. Since dehydrogenases occur in soil in all livingmicrobial cells, the DHA test is used as an indicator of the overall soil microbial activity, or a measure of the general soil metabolic activity. A close relationship has been observed between DHA and soil organic matter content, microbial biomass, population size of soil microorganisms, respiration, denitrification, and other indicators of soil microbial activity. Additionally, dehydrogenase activity is often used as a measure of any disruption caused by pesticides, trace elements, etc., or management practices to the soil.

Procedure

1. Weight 6 g soil sample in sterile screw cap tubes in triplicates along with blank (without soil). This will result in 3 vials with soil and 1 blank (without soil).
2. Add 1ml of 3% TTC and 2.5 ml deionized water to each of the vials, including the blank. Mix the soil and liquid with a glass stirring rod, and then put caps on the vials (the vials need to be sealed against oxygen from the air). A small amount of free liquid should just appear at the soil surface. Incubate the vials at 30°C for 24 h.
3. To each vial, add 10 ml of methanol, stir, and transfer the suspension to a funnel fitted with Whatman no. 5 filter paper. Collect the filtrate in a 50 ml Erlenmeyer flask. [Precaution: During this procedure, it is necessary to keep the sample wet at all times until extraction is complete to avoid air drawn through the soil].
4. Wash the vial and the funnel containing the sediment with two more 10 ml methanol rinses until the filtrate is clear of red colour. Add more methanol to bring the total volume to 50 ml.
5. If suspended soil particles are present in tubes, centrifuge the filtrate at 14,000 rpm for 10 min before reading absorbance.
6. Transfer 5 ml of each sample to a cuvette, read the absorbance of each sample on a spectrophotometer at 485 nm using the blank as the zero. Rinse the cuvettes with 1–2 ml rinses of methanol between samples.
7. Calculate the amount of TPF produced by reference to a calibration graph prepared from TPF standards. (To prepare this graph, dilute 10 mL of TPF standard solution to 100 mL with methanol

(100 3g of TPF/mL) Pipette 5, 10, 15, 20, 25 and 30 mL aliquots of this solution into 50 mL with methanol and mix thoroughly. Measure the intensity of the red colour of TPF as described for samples. Plot the absorbance readings against the amount of TPF in 50 mL standard solutions).

8. Express the results as g TPF g^{-1} dry soil. Report the average of the triplicates.

Calculation

Standard curve data and equation for (TPF) using visible absorption spectrophotometry at l = 485 nm. A is the absorbance value. X = the concentration of TPF analyzed in the solution analyzed in the spectrophotometer in mg mL^{-1}

X (TPF) in mg mL^{-1}	Absorbance
0.00	0.000
5.0	0.214
10	0.423
15	0.642
20	0.833
25	1.051
30	1.244

Example for calculations

Soil	**Absorbance**	**Average**
Soil 1	0.189	
Soil 1	0.214	0.202
Soil 1	0.205	
Soil 2	0.226	
Soil 2	0.218	0.228
Soil 2	0.240	

Soil 3	0.167	
Soil 3	0.193	0.170
Soil 3	0.151	

Solution:

$$X = \frac{A - 0.00629}{0.0415}$$

$$\therefore X = \frac{0.202 - 0.00629}{0.0415}$$

$$\therefore X = 4.71\ \mu g/ml \text{ of extractant}$$

$$\therefore X = \frac{4.71 \times 50}{6} g^{-1} \text{ soil}$$

$$X = 39.25 \mu g \text{ TPF } g^{-1} \text{soil}$$

EXPERIMENT-7

Isolation of Rhizobium from root nodules

Aim

To isolate Rhizobium isolates from root nodules of leguminous plants.

Materials Required

Petri dishes, 1-ml pipettes 10-ml pipette, test tubes, conical flasks, 500 ml beaker, glass spreader, stainless steel pipette case, ethyl alcohol, 0.1N hydrochloric acid (HCI), 0.1N sodium hydroxide (NaOH), distilled water, rhizobium agar, groundnut plant with root nodules, pH meter, pestle and mortar, bunsen burner, autoclave, incubator, laminar flow chamber, turntable.

Background

Leguminous plants including peas and beans rely upon symbiotic relationships with nitrogen-fixing bacteria. These bacteria live freely in the soil or within nodules in the root system, and produce nitrogen compounds that are utilized by the plant. In poor soils, supplementing leguminous crops with cultured nitrogen-fixing bacteria from soils may boost the growth and health of the plants. This can result in bigger, hardier plants, which in turn give greater crop yield.

Procedure

1. Ground nut, *Arachis hypogaea* plant was carefully uprooted and the root system was washed under running water to remove the adhesive soil particles. The colour of the nodules varied from brown to pink depending on the state of pigment present in them. For experiment healthy unbroken pink nodules were selected.
2. The sterilizing agent, 0.1 % mercuric chloride was used. The nodules were immersed in sterilizing agents for 4-5 mints and then washed repeatedly with sterile distilled water.
3. Then they are washed in 70% ethyl-alcohol followed by washing with sterile distilled water again. Nodules were washed in a small aliquot of sterile distilled water with the help of a glass rod.
4. The ingredients of rhizobium agar medium is weighed and dissolved in of distilled water in conical flask by shaking and swirling and sterilized by autoclaving.

Composition Ingredients Gms/Litre

Mannitol	10.000
Dipotassium phosphate	0.500
Magnesium sulphate	0.6
Yeast extract	1.000
Sodium chloride	0.100
Agar	20.000
pH	6.8

5. To prepare agar plates, before the sterilised rhizobium agar medium cools and solidifies, in warm molten condition, it is poured aseptically into the sterilised Petri dishes, so that the molten medium covers the bottom of the Petri dishes completely. Then, the plates are covered with their lids and allowed to cool, so as to solidify the medium in them.
6. Ten fold serial dilution of nodular extract was prepared by taking 1g of nodular extract into 10 ml of sterile distilled water and mixed well to get nodular extract suspension.
7. One ml of nodular extract suspension was diluted with 9 ml of sterile distilled water making the dilution to 10^{-2}. Repeat this dilution step, each time with 1 mL of the previous suspension and 9-mL deionised water blank. Label these sequentially, this results in serial dilutions of 10^{-2} through 10^{-7} the final dilution.
8. Suspension (0.1 ml) of nodular extract prepared from each dilution was inoculated into sterile rhizobium agar plates as indicated 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions to be used for isolation of rhizobium. The sample was speeded throughout the rhizobium agar plates and inoculated Petri plates were incubated for 4-7 days in an incubator at 25-30°C.

Observation

At 3-5 days of the incubation, the Rhizobium growth was observed.

Result

On Rhizobium agar medium mucoid, flat, watery and translucent colonies was observed on 3 days of incubation at room temperature 25-30°C.